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(54) Title: G PROTEIN COUPLED RECEPTOR

(57) Abstract: The invention provides nucleic acid encoding a G protein coupled receptor (adenine binding GPCR) with specificity for adenine. isolated adenine binding GPCR, assay and treatment methods utilising the same.

G PROTEIN COUPLED RECEPTORField of the Invention

5 The present invention relates to a novel mammalian G protein coupled receptor, to nucleic acid encoding it and to its use in assays.

Background to the Invention

10

GTP-binding proteins (G proteins) act as intermediaries between binding of ligands such as hormones and other chemical mediators to G protein coupled receptors (GPCRs) and activation of intracellular effectors. Upon binding of  
15 a ligand to a GPCR, the cytoplasmic domains of the receptor undergo conformational changes which enable interaction of the receptor with a G protein, which in turn enables activation of intracellular intermediaries such as adenylate cyclase, phospholipase C or ion  
20 channels. Such a system allows amplification of the original signal as many second messengers can be produced in response to the binding of a single ligand at the GPCR. Through this mechanism, cells are able to sense and respond to alterations in their external environment.

25

G protein coupled receptors form a superfamily of integral plasma membrane proteins, each receptor sharing the common feature of seven hydrophobic transmembrane domains, each of which is 20 -30 amino acids long and which are linked  
30 by hydrophilic amino acid sequences of varied length. The amino terminus of the receptor is extracellular with the carboxy terminus found in the cytoplasm of the cell.

GPCRs are found in a wide range of tissues and cell types  
35 and are involved in many different physiological

processes. They are activated by a broad range of ligands, for example, hormones such as luteinizing hormone, follicle stimulating hormone, chorionic gonadotrophin, thyrotropin, adrenocorticotrophin, glucagon and

5 vasopressin; neurotransmitters such as 5-HT, acetylcholine (muscarinic AchR), histamine, prostaglandins, calcitonin, leukotrienes and  $\text{Ca}^{2+}$ . The broad distribution and wide variety of roles of GPCRs indicate that GPCRs may play important roles in a variety of pathological conditions.  
10 Indeed, GPCRs have been found to be involved in diseases related to bronchoconstriction, hypertension, inflammation, hormonal disturbance, diabetes, apoptosis, nociception, facilitation of neurotransmission and tremor disorders.

15

One group of GPCRs of particular interest are those which act as receptors for nucleotides. Nucleotide receptors are structured into three families: P1, P2Y and P2X (Ralevic and Burnstock, 1998 Pharmacological Reviews 50(3):413-  
20 492), of which the P1 and P2Y families are GPCRs. P1 receptors are activated by adenosine and do not bind adenine (Bruns et al., Proc. Natl. Acad. Sci., USA, 77(50):5547-51, 1980; Schwabe and Trost, 1980 Naunyn Schmiedeberg's Arch Pharmacol. 313(3):197-87; Schwabe et  
25 al., 1982, Naunyn Schmiedeberg's Arch Pharmacol. 321(1):84-87). Members of the P1 receptor family are divided into three subfamilies, known as the adenosine type 1 family, the adenosine type 2 family and the adenosine type 3 family.

30

The P2Y family contains four families of GPCRs activated by nucleotides. In detail, these are P2Y1 (activated by ADP and ATP), P2Y2 (also called P2U, these GPCRs are activated at equal potency by ATP and UTP), P2Y3 and P2Y6  
35 (relative agonist strength is  $\text{UDP} > \text{UTP} > \text{ADP}$ ) and P2Y4

(UTP>ATP). A number of other GPCRs having sequence homology to the P2Y2 group were initially thought to be nucleotide receptors as well (P2Y5, P2Y7, P2Y9 and P2Y10), but are now known not to be activated by nucleotides.

5

The P2X family is a group of unrelated proteins that is not linked to G proteins. Members of this group are ligand gated ion channels.

10 Because G protein coupled receptors play such an important role in a wide variety of cellular and physiological processes, there is ongoing interest in assessing the function of such receptors and their potential roles in a variety of diseases.

15.

#### Summary of the Invention

In the course of research into GPCRs, the present inventors have discovered a novel GPCR, which represents a new class of GPCR and which has been termed adenine binding GPCR. When the sequence of the novel GPCR in the rat was compared with those available in the prior art, the inventors found to their surprise that the closest related GPCR shares only approximately 50% sequence identity with the novel receptor, suggesting that the novel GPCR represents a member of a new class of GPCR. Further studies identified the natural ligand of the receptor to be adenine. This is the first demonstration of a GPCR which has adenine as its natural ligand, suggesting that the GPCR represents a new class of GPCR. Indeed, this is the first demonstration of a receptor of any kind which has adenine as its natural receptor and, as the novel receptor has been found to be present in the mammalian cortex, represents the first disclosure of adenine as a neurotransmitter. Further studies identified six human

35

homologues of the receptor, which also form part of the invention.

The present invention provides an adenine binding G  
5 protein coupled receptor. In particular, the present  
invention provides an isolated polypeptide having the  
sequence of SEQ ID NO:2 or SEQ ID NO:4. Polypeptides which  
are fragments of said polypeptide of SEQ ID NO:2 or SEQ ID  
10 NO:4 are also provided, said fragments being of at least  
10, for example at least 20, 30 40, 50, 75, 100 or 150 or  
more amino acids in size. Such fragments may be derived  
from the N-terminal region of SEQ ID NO:2 or SEQ ID NO:4  
respectively. Fragments including the N-terminal region  
may be used to reconstitute the extracellular portion of  
15 the receptor to provide receptor binding sites.  
Preferably, fragments will retain the ability to bind  
adenine.

In another embodiment, the invention provides an isolated  
20 polypeptide having at least 50% and preferably at least  
60%, 70%, 80%, 90%, 95% or 98% sequence identity to the  
polypeptide of SEQ ID NO:2 or SEQ ID NO:4. Such  
polypeptides desirably retain the ability to bind adenine  
and preferably to form a receptor that can be activated by  
25 adenine. Similarly, polypeptides which are fragments of at  
least 20 amino acids of these polypeptides preferably  
having at least 50% and preferably at least 60%, 70%, 80%,  
90%, 95% or 98% identity to a fragment of SEQ ID NO:2 or  
SEQ ID NO:4 of corresponding length, including N-terminal  
30 fragments, are provided. Such polypeptides desirably  
retain the ability to bind adenine and preferably to form  
a receptor that can be activated by adenine.

The present invention also provides an isolated nucleic  
35 acid sequence encoding the polypeptide of SEQ ID NO:2 or

SEQ ID NO:4. In a preferred aspect, the isolated sequence is that of SEQ ID NO:1 or SEQ ID NO:3. The invention also provides a DNA molecule encoding the polypeptide of SEQ ID NO:2 or SEQ ID NO:4. Also provided by the invention are  
5 isolated nucleic acids encoding the polypeptides mentioned in the previous two paragraphs. Further provided are DNA molecules encoding said polypeptides.

Nucleic acids of the invention further include nucleic  
10 acids which comprise a sequence having at least 50% and preferably at least 60%, 70%, 80%, 90%, 95% or 98% sequence identity to the nucleic acid sequences of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO:5, 6,7,8 or 9 or their complements. Preferably, these sequences will hybridise to  
15 the corresponding nucleic acid under conditions controlled to minimise non-specific binding. Preferably stringent to moderately stringent hybridisation conditions are preferred. Suitable conditions include, e.g. for detection of sequences that are about 80-90% identical,  
20 hybridization overnight at 42°C in 0.25M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na<sub>2</sub>HPO<sub>4</sub>,  
25 pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

It will be appreciated that such nucleic acids do not necessarily encode "full length" polypeptides, and will  
30 thus include nucleic acids which represent, for example, mutant forms of the adenine binding GPCR gene in which the coding sequence has been prematurely terminated by either a substitution resulting in a stop codon or a frameshift mutation. These are also nucleic acids of the invention.

The invention also provides nucleic acids which are fragments of the nucleic acids encoding a polypeptide of the invention. In one aspect, the invention provides  
5 nucleic acids primers which consist essentially of from 15 to 50, for example from 15 to 35, 18 to 35, 15 to 24, 18 to 30, 18 to 21 or 21 to 24 nucleotides of a sequence encoding a polypeptide of the invention or its complement.

10 Nucleic acids and polypeptides of the invention may be used therapeutically to treat disease. In particular, they may be used to treat diseases, the pathology of which is associated with action at purine receptors, particularly those associated with mutation or downregulation of  
15 expression of native adenine binding GPCRs. In specific, they may be used as anticonvulsives, sedatives, hypnotics, hypotensives, antiarrhythmics, negative chronotropics, dromotropics and inotropics, or in the treatment of pathological tremor disorders, ataxia, or of disease  
20 associated with nociception or the facilitation of neurotransmission. Furthermore, as well as finding use in disorders associated with the CNS, they may be used as inhibitors of platelet aggregation, stimulators of NO production by vascular endothelial cells in the  
25 cardiovascular system, as inhibitors of gastric secretion or in disease associated with apoptosis, bronchoconstriction, vasodilation or inflammation.

In a further aspect, there are provided vectors comprising  
30 the sequences of said nucleic acids, particularly expression vectors comprising a promoter operably linked to the nucleic acid sequences of the invention. The vectors may be carried by a host cell, and expressed within said cell. Following said expression, polypeptides  
35 of the invention may be recovered.

In a further aspect, the invention provides assay methods for the identification of substances which bind to or modulate the activity of polypeptides of the invention. In particular it is envisaged that such substances which may be peptide or non-peptide be used in methods of treatment as described above.

As the present inventors are the first to identify a receptor of any kind which has adenine as its natural ligand and, as the novel receptor has been found to be present in the mammalian cortex, the inventors are the first to disclose adenine as a neurotransmitter. Therefore, the present invention opens up the possibility of using adenine itself and/or compounds mimicking or antagonising adenine in therapeutic applications. Therefore the invention further extends to adenine for use in a method of treatment of a human or animal body. In particular, it is envisaged that adenine be used for treatment of disease, the pathology of which is associated with action at purine receptors, particularly adenine receptors. Furthermore, the invention extends to the use of adenine in the preparation of a medicament for the treatment of any of the diseases or conditions described above.

The invention also provides antibodies and binding fragments thereof capable of selectively binding to polypeptides of the invention. The antibodies and binding fragments may therefore be used in diagnostic tests for the identification of the polypeptides of the invention in biological samples.

These and other aspects of the invention are described herein in more detail.



Description of sequences.

SEQ ID NO:1 is the nucleotide sequence for rat adenine  
5 binding GPCR.

SEQ ID NO:2 is the amino acid sequence for rat adenine  
binding GPCR.

10 SEQ ID NO:3 is the nucleotide sequence for human adenine  
binding GPCR1.

SEQ ID NO:4 is the amino acid sequence for human adenine  
binding GPCR1.

15

SEQ ID NO:5 is the nucleotide sequence for human adenine  
binding GPCR2.

20 SEQ ID NO:6 is the nucleotide sequence for human adenine  
binding GPCR3.

SEQ ID NO:7 is the nucleotide sequence for human adenine  
binding GPCR4.

25 SEQ ID NO:8 is the nucleotide sequence for human adenine  
binding GPCR5.

SEQ ID NO:9 is the nucleotide sequence for human adenine  
binding GPCR6.

30

Brief Description of the Drawings

Figure 1 illustrates sequence alignment of the amino acid  
sequences of the rat adenine binding GPCR (SEQ ID NO:2)  
35 and human adenine binding GPCR1 (SEQ ID NO:4)

Figure 2 illustrates activation of rat adenine binding GPCR by porcine cortex extract C18 fractions after transient transfection into HEK293 cells expressing the  
5 Gαqo chimera.

Figure 3 illustrates activation of rat adenine binding GPCR by porcine cortex extract C18 fractions after transient transfection into HEK293 cells expressing the  
10 Gαqi chimera.

Figure 4 illustrates the stimulation of Ca<sup>2+</sup> transients in HEK293/Gαqi cells transfected with rat adenine binding GPCR/pcDNA3 by application of 3.3nM to 33μM adenine.  
15

Figure 5 illustrates Ca<sup>2+</sup> transients elicited in HEK293/Gαqi cells transfected with rat adenine binding GPCR/pcDNA3 by application of 3.3μM uridine, guanine, hypoxanthine, xanthine, caffeine, UDP, and adenine.  
20

Figure 6 illustrates Ca<sup>2+</sup> transients elicited in HEK293/Gαqi cells transfected with rat adenine binding GPCR/pcDNA3 by application of 3.3•M adenosine, AMP, ADP, ATP and adenine.  
25

Figure 7 illustrates Ca<sup>2+</sup> transients elicited in HEK293/Gαqi cells transfected with rat adenine binding GPCR/pcDNA3 by application of 3•M PBS, xanthine, guanine and hypoxanthine.  
30

Figure 8a illustrates stimulation of [<sup>35</sup>S]GTPγS binding on CHO membranes, transiently transfected with the rat adenine binding GPCR. Data are presented as the mean of 3

independent experiments ( $\pm$ SEM; n=3).

Figure 8b illustrates stimulation of [ $^{35}$ S]GTP $\gamma$ S binding on wild type CHO membranes. Data are presented as the mean  
5 of 3 independent experiments ( $\pm$ SEM; n=3).

Figure 9a illustrates inhibition of forskolin stimulated cAMP formation on CHO cells, transiently transfected with the rat adenine binding GPCR. The assay was performed in  
10 0.5% hiFCS, using the direct RIA kit from NEN.  
Representative curves.

Figure 9b illustrates inhibition of forskolin stimulated cAMP formation on CHO cells, transiently transfected with  
15 the rat adenine binding GPCR. The assay was performed in 2% hiFCS, using the direct RIA kit from NEN.  
Representative curves.

Figure 10 illustrates adenine dose response curve for the  
20 stimulation of [ $^{35}$ S]GTP $\gamma$ S binding on CHO membranes, permanently transfected with the rat adenine binding GPCR.

Figure 11a illustrates inhibition of forskolin stimulated cAMP formation on CHO cells, permanently transfected with  
25 the rat adenine binding GPCR (clone 28). The figure shows representative curves obtained using 0.5 and 10% serum concentrations, each in the presence and absence of Na-butyrate induction.

30 Figure 11b illustrates inhibition of forskolin stimulated cAMP formation on wild type CHO cells. The figure shows representative curves obtained using 0.5 and 10% serum concentrations, each in the presence and absence of Na-butyrate induction.

Figure 12a illustrates adenine dose response curves of intracellular  $\text{Ca}^{2+}$  measurements on CHO cells, permanently transfected with the rat adenine binding GPCR (clone 28) in the presence and absence of Na-byturate.

Figure 12b illustrates adenine dose response curves of intracellular  $\text{Ca}^{2+}$  measurements on wild type CHO cells, in the presence and absence of Nabutyarte.

10

### Detailed Description of the Invention

#### Nucleic acid

15 Nucleic acid includes DNA (including both genomic and cDNA) and RNA. Where nucleic acid according to the invention includes RNA, reference to the sequences shown in the accompanying listings should be construed as reference to the RNA equivalent, with U substituted for T.

20

Nucleic acid of the invention may be single or double stranded. Single stranded nucleic acids of the invention include anti-sense nucleic acids. Thus it will be understood that reference to SEQ ID NO:1 or sequences comprising SEQ ID NO:1 or fragments thereof include complementary sequences unless the context is clearly to the contrary. The same applies to SEQ ID NOs:3,5,6,7,8 and 9.

30 Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human  
35 genome, except possibly one or more regulatory sequence(s)

for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA.

The invention also provides nucleic acids which are  
5 fragments of the nucleic acids encoding a polypeptide of the invention. In one aspect, the invention provides nucleic acids primers which consist essentially of from 15 to 50, for example from 15 to 35, 18 to 35, 15 to 24, 18 to 30, 18 to 21 or 21 to 24 nucleotides of a sequence  
10 encoding a polypeptide of the invention or its complement.

The term "consist essentially of" refers to nucleic acids which do not include any additional 5' or 3' nucleic acid sequences. In a further aspect of the invention, nucleic  
15 acids of the invention which consist essentially of from 15 to 30 nucleotides as defined above may however be linked at the 3' but preferably 5' end to short (e.g from 4 to 15, such as from 4 to 10 nucleotides) additional sequences to which they are not naturally linked. Such  
20 additional sequences are preferably linkers which comprise a restriction enzyme recognition site to facilitate cloning when the nucleic acid of the invention is used for example as a PCR primer.

25 Primers of the invention are desirably capable of selectively hybridising to nucleic acids encoding the polypeptides of the invention. By "selective", it is meant selective with respect to sequences encoding other purine receptors and in particular with respect to receptors  
30 other than adenine receptors. The ability of the sequence to hybridise selectively may be determined by experiment or calculated.

For example, one way to calculate  $T_m$  of a primer is by  
35 reference to the formula for calculating the  $T_m$  of primers

to a homologous target sequence. This formula is  $T_m(^\circ\text{C}) = 2(A+T) + 4(G+C) - 5$ . This will provide the  $T_m$  under conditions of 3xSSC and 0.1% SDS (where SSC is 0.15M NaCl, 0.015M sodium citrate, pH 7). This formula is generally  
5 suitable for primers of up to about 50 nucleotides in length. In the present invention, this formula may be used as an algorithm to calculate a nominal  $T_m$  of a primer for a specified sequence derived from a sequence encoding a polypeptide of the invention. The  $T_m$  may be compared to  
10 a calculated  $T_m$  for GPCR sequences of humans and rats, based upon the maximum number of matches to any part of these other sequences.

Suitable conditions for a primer to hybridise to a target  
15 sequence may also be measured experimentally. Suitable experimental conditions comprise hybridising a candidate primer to both nucleic acid encoding a polypeptide of the invention and nucleic acid encoding other adenine receptors on a solid support under low stringency  
20 hybridising conditions (e.g. 6xSSC at 55 $^\circ\text{C}$ ), washing at reduced SSC and/or higher temperature, for example at 0.2xSSC at 45 $^\circ\text{C}$ , and increasing the hybridisation temperature incrementally to determine hybridisation  
25 conditions which allow the primer to hybridise to nucleic acid encoding a polypeptide of the invention but not other purine receptor encoding nucleic acids.

Nucleic acids of the invention, particularly primers, may carry a revealing label. Suitable labels include  
30 radioisotopes such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , fluorescent labels, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the invention and may be detected using by techniques known per se.

Primers of the present invention may be comprised of synthetic nucleic acids, such as those with modified backbone structures intended to improve stability of the nucleic acid in a cell. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the in vivo activity or lifespan of polynucleotides of the invention.

Also included within the scope of the invention are antisense sequences based on the nucleic acid sequences described herein, preferably in the form of oligonucleotides, particularly stabilized oligonucleotides, or ribozymes.

Antisense oligonucleotides may be designed to hybridise to the complementary sequence of nucleic acid, pre-mRNA or mature mRNA, interfering with the production of polypeptide encoded by a given target DNA sequence, so that its expression is reduced or prevented altogether. Ribozymes will be designed to cleave mRNA encoded by an adenine binding GPCR encoding nucleic acid sequence of the invention, desirably at a target sequence specific to the adenine binding GPCR, i.e one which is not common to other GPCR sequences. The construction of antisense sequences and their use is described in Peyman and Ulman, Chemical Reviews, 90:543-584, (1990), Crooke, Ann. Rev. Pharmacol. Toxicol., 32:329-376, (1992), and Zamecnik and Stephenson, P.N.A.S, 75:280-284, (1974). The construction

of ribozymes and their use is described in for instance Gibson and Shillitoe, Molecular Biotechnology 7(2): 125-137, (1997).

5 Antisense and ribozyme sequences of the invention may be introduced into mammalian cells lines in culture to study the function of adenine binding GPCR, for example by causing down-regulation of this gene and observing phenotypic effects, or the expression or location of  
10 proteins described herein which associate with adenine binding GPCR. In cells where aberrant expression of adenine binding GPCR occurs, such antisense and ribozyme sequences may be used to down-regulate the expression of the gene.

15

The cDNA sequence of the GPCR of the invention may be cloned using standard PCR (polymerase chain reaction) cloning techniques. This involves making a pair of primers to 5' and 3' ends on opposite strands of SEQ ID  
20 NO:1, bringing the primers into contact with mRNA or cDNA obtained from a mammalian cortical cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture  
25 on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector. The same applies to SEQ ID NOS:3,5,6,7,8 and 9.

30

Polynucleotides which are not 100% homologous to the sequence of SEQ ID NO:1 or SEQ ID NO:3 but which encode either SEQ ID NO:2 or SEQ ID NO:4 or other polypeptides of the invention can be obtained in a number of ways.

35



For example, site directed mutagenesis of the sequence of  
SEQ ID NO: 1 or SEQ ID NO:3 may be performed. This is  
useful where for example silent codon changes are required  
to sequences to optimise codon preferences for a  
5 particular host cell in which the polynucleotide sequences  
are being expressed. Other sequence changes may be  
desired in order to introduce restriction enzyme  
recognition sites, or to alter the property or function of  
the polypeptides encoded by the polynucleotides. Further  
10 changes may be desirable to represent particular coding  
changes which are required to provide, for example,  
conservative substitutions.

Nucleic acids of the invention may comprise additional  
15 sequences at the 5' or 3' end. For example, synthetic or  
natural 5' leader sequences may be attached to the nucleic  
acid encoding polypeptides of the invention. The  
additional sequences may also include 5' or 3'  
untranslated regions required for the transcription of  
20 nucleic acid of the invention in particular host cells.

In addition, other animal, particularly mammalian (e.g.  
humans or rabbits), more particularly primate including  
human, homologues of adenine binding GPCR may be obtained.  
25 Such sequences may be obtained by making or obtaining  
cDNA libraries made from dividing cells or tissues or  
genomic DNA libraries from other animal species, and  
probing such libraries with probes comprising all or part  
of SEQ ID NO:1 or of SEQ ID NO:3 under conditions of  
30 medium to high stringency (for example 0.03M sodium  
chloride and 0.03M sodium citrate at from about 50°C to  
about 60°C).

The present invention further extends to an isolated DNA  
35 sequence comprising sequences encoding a polypeptide of

the invention but in which the encoding sequences are divided up into two or more (preferably no more than five, e.g. four or three) exons. Such exon sequences may be natural and obtained from genomic clones, or synthetic.

5 Exon sequences may be used in the construction of mini-gene sequences which comprise nucleic acid encoding polypeptides of the invention which sequences are interrupted by one or more exon sequences.

10 Mini-genes may also be constructed using heterologous exons, derived from any eukaryotic source.

Nucleic acid according to the present invention, such as a full-length coding sequence or oligonucleotide probe or

15 primer, may be provided as part of a kit, e.g. in a suitable container such as a vial in which the contents are protected from the external environment. The kit may include instructions for use of the nucleic acid, e.g. in PCR and/or a method for determining the presence of

20 nucleic acid of interest in a test sample. A kit wherein the nucleic acid is intended for use in PCR may include one or more other reagents required for the reaction, such as polymerase, nucleosides, buffer solution etc.

25 The nucleic acid of the invention may be used in nucleic acid-based tests for detecting the adenine binding GPCR encoding sequences in rat, human or other mammalian body or tissues or samples obtained therefrom. In the case of detecting, this may be qualitative and/or quantitative,

30 including such methods as microarray technology on a DNA chip. Detection includes analytical steps such as those which involve sequencing the gene in full or in part.

Such tests for detecting generally comprise bringing a

35 human sample containing DNA or RNA into contact with a

probe comprising a nucleic acid of the invention or primer of the invention under hybridizing conditions and detecting any duplex formed between the probe and nucleic acid in the sample. Such detection may be achieved using techniques such as PCR or by immobilizing the probe on a solid support, removing nucleic acid in the sample which is not hybridized to the probe, and then detecting nucleic acid which has hybridized to the probe. Alternatively, the sample nucleic acid may be immobilized on a solid support, and the amount of probe bound to such a support can be detected. Suitable assay methods of this any other formats can be found in for example WO89/03891 and WO90/13667.

A further method of detection according to the invention is in detecting changes to wild-type adenine binding GPCR genes, including single base changes, for example using single stranded conformational polymorphism (SSCP) analysis. Nucleic acid sequence from all or part of an adenine binding GPCR encoding DNA or mRNA in a sample may be hybridized to a reference sequence, and the mobility of the hybrid is observed in a gel under conditions where any non-hybridized regions within the duplex give rise to changes in mobility.

The nucleic acids of the present invention are also useful in tissue distribution studies, to confirm and extend the knowledge of this gene's distribution in the cortex, dorsal root, CNS and PNS and other tissues.

### Polypeptides.

Isolated polypeptides of the invention will be those as defined above in isolated form, free or substantially free of material with which it is naturally associated such as

other polypeptides with which it is found in the cell. The polypeptides may of course be formulated with diluents or adjuvants and still for practical purposes be isolated - for example the polypeptides will normally be mixed with  
5 gelatin or other carriers if used to coat microtitre plates for use in immunoassays. The polypeptides may be glycosylated, either naturally or by systems of heterologous eukaryotic cells, or they may be (for example if produced by expression in a prokaryotic cell)  
10 unglycosylated. Polypeptides may be phosphorylated and/or acetylated.

A polypeptide of the invention may also be in a substantially purified form, in which case it will  
15 generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention.

20 Polypeptides of the invention may be modified for example by the addition of histidine residues to assist their purification or by the addition of a signal sequence to promote their secretion from a cell.

25 Polypeptides having at least 50%, for example 60%, 70%, 80%, 90%, 95% or 98% sequence identity to SEQ ID NO:2 or SEQ ID NO:4 may be polypeptides which are amino acid sequence variants, alleles, derivatives or mutants of SEQ ID NO:2 or SEQ ID NO:4 respectively, and are also provided  
30 by the present invention. For example such a polypeptide may have an amino acid sequence which differs from that given in SEQ ID NO:2 or SEQ ID NO:4 by one or more of addition, substitution, deletion and insertion of one or more (such as from 1 to 20, for example 2, 3, 4, or 5 to  
35 10) amino acids.

The percentage identity of polypeptide sequences can be calculated using commercially available algorithms which compare a reference sequence( e.g. SEQ ID NO:2 or SEQ ID NO:4 of the present invention) with a query sequence. Further details of assessing identity are described below.

In the present study, the nucleotide sequence SEQ ID NO:2 was analysed by BLAST (Altschul et al., 1997) search to examine whether related GPCRs with known ligands could be found. However the closest homologues of this orphan GPCR that were retrieved, were orphan GPCRs themselves. These were a family of human GPCRs (Derwent sequence database accession numbers Z10067, Z10068, Z10069, Z10070, Z10071 and Z10072) cloned from dorsal root ganglion that have approximately 50% of residues in common with the rat adenine binding GPCR. The present inventors have now identified these six orphan GPCRs as being six human homologues of the rat adenine binding GPCR. The six homologues have been termed human adenine binding GPCR 1, 2, 3, 4, 5 and 6 respectively. The next closest homologue is the mas related gene which shares 33% of residues with adenine binding GPCR.

Where a query sequence is determined to have an identity to that of SEQ ID NO:2 or SEQ ID NO:4 of at least 50% and preferably at least 60%, 70%, 80%, 90%, 95% or 98% said sequence being that of a polypeptide retaining adenine binding receptor activity, such a sequence forms part of the present invention.

A polypeptide according to the present invention may be isolated and/or purified (e.g. using an antibody) for instance after production by expression from encoding nucleic acid. The isolated and/or purified polypeptide

may be used in formulation of a composition, which may include at least one additional component, for example a pharmaceutical composition including a pharmaceutically acceptable excipient, vehicle or carrier.

5

A polypeptide according to the present invention may be used as an immunogen or otherwise in obtaining specific antibodies. Antibodies are useful in purification and other manipulation of polypeptides, diagnostic screening and therapeutic contexts. This is discussed further below.

A polypeptide according to the present invention may be used in screening for molecules which bind to it or modulate its activity or function. Such molecules may be useful in a therapeutic (possibly including prophylactic) context.

A polypeptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes, e.g.  $^{125}\text{I}$ , enzymes, antibodies, polynucleotides and linkers such as biotin. Labelled polypeptides of the invention may be used in diagnostic procedures such as immunoassays in order to determine the amount of a polypeptide of the invention in a sample. Polypeptides or labelled polypeptides of the invention may also be used in serological or cell mediated immune assays for the detection of immune reactivity to said polypeptides in animals and humans using standard protocols.

A polypeptide or labelled polypeptide of the invention or fragment thereof may also be fixed to a solid phase, for example the surface of an immunoassay well or dipstick.

Such labelled and/or immobilized polypeptides may be packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

5

Such polypeptides and kits may be used in methods of detection of antibodies to such polypeptides present in a sample or active portions or fragments thereof by immunoassay.

10

Immunoassay methods are well known in the art and will generally comprise:

- (a) providing a polypeptide comprising an epitope bindable by an antibody against said protein;
- 15 (b) incubating a biological sample with said polypeptide under conditions which allow for the formation of an antibody-antigen complex; and
- (c) determining whether antibody-antigen complex comprising said polypeptide is formed.

20

### Sequence Identity

The percentage identity of nucleic acid and polypeptide sequences can be calculated using commercially available  
25 algorithms which compare a reference sequence with a query sequence. The following programs (provided by the National Center for Biotechnology Information) may be used to determine homologies/identities: BLAST, gapped BLAST, BLASTN and PSI-BLAST, which may be used with default  
30 parameters.

The algorithm GAP (Genetics Computer Group, Madison, WI) uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches  
35 and minimizes the number of gaps. Generally, the default

parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4.

Another method for determining the best overall match  
5 between a nucleic acid sequence or a portion thereof, and a query sequence is the use of the FASTDB computer program based on the algorithm of Brutlag et al (Comp. App. Biosci., 6; 237-245 (1990)). The program provides a global sequence alignment. The result of said global  
10 sequence alignment is in percent identity. Suitable parameters used in a FASTDB search of a DNA sequence to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size  
15 Penalty=0.05, and Window Size=500 or query sequence length in nucleotide bases, whichever is shorter. Suitable parameters to calculate percent identity and similarity of an amino acid alignment are: Matrix=PAM 150, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization  
20 Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty=0.05, and Window Size=500 or query sequence length in nucleotide bases, whichever is shorter.

### Antibodies

25 The provision of the polypeptides of the invention enables for the production of antibodies able to bind rat or human adenine binding GPCR in a specific manner. Thus the invention provides an antibody which is able to bind  
30 specifically to a polypeptide of the invention and not to other purinergic GPCRs. Such an antibody will have an affinity for a polypeptide of the invention of at least 100 fold, preferably at least 1000 fold more than to other purinergic GPCRs. Such antibodies may be produced using  
35 epitopes of polypeptides of the invention which are not



present in other GPCRs. Such epitopes can be determined by seeking differences between polypeptides of the invention and other purinergic GPCR sequences.

5 Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit) with a polypeptide of the invention. Antibodies may be obtained from immunised animals using any of a variety of  
10 techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, Nature, 357:80-82, 1992).

15 As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using  
20 lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Antibodies according to the present invention may be  
25 modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies,  
30 including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen  
35 or other binding partner are the Fab fragment consisting

- of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain;  
5 isolated CDR regions and F(ab')<sub>2</sub> fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.
- 10 The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The  
15 linkage of reporter molecules may be direct or indirect, covalent, e.g. via a peptide bond or non-covalent. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.
- 20 The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.
- 25 Antibodies according to the present invention may be used in screening for the presence of a polypeptide, for example in a test sample containing cells or cell lysate as discussed, and may be used in purifying and/or  
30 isolating a polypeptide according to the present invention, for instance following production of the polypeptide by expression from encoding nucleic acid therefor. Antibodies may modulate the activity of the polypeptide to which they bind and so, if that polypeptide  
35 has a deleterious effect in an individual, may be useful

in a therapeutic context (which may include prophylaxis).

An antibody may be provided in a kit, which may include instructions for use of the antibody, e.g. in determining the presence of a particular substance in a test sample. One or more other reagents may be included, such as labelling molecules, buffer solutions, elutants and so on. Reagents may be provided within containers which protect them from the external environment, such as a sealed vial.

10

### Vectors

Nucleic acid sequences of the present invention may be incorporated into vectors, particularly expression vectors. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

25

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector.

30

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding

35

sequence is achieved under condition compatible with the control sequences.

Suitable vectors can be chosen or constructed, containing  
5 appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. phage, phagemid or baculoviral, cosmids, YACs, BACs,  
10 or PACs as appropriate. Vectors include gene therapy vectors, for example vectors based on adenovirus, adeno-associated virus, retrovirus (such as HIV or MLV) or alpha virus vectors.

15 The vectors may be provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter.

The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the  
20 case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used in vitro, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used in vivo, for example in methods of gene therapy.

25 Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a  
30 heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others.

Promoters and other expression regulation signals may be  
35 selected to be compatible with the host cell for which the

expression vector is designed. For example, yeast promoters include *S. cerevisiae* GAL4 and ADH promoters, *S. pombe* nmt1 and adh promoter. Mammalian promoters include the metallothionein promoter which can be induced  
5 in response to heavy metals such as cadmium. Viral promoters such as the SV40 large T antigen promoter or adenovirus promoters may also be used. All these promoters are readily available in the art.

10 The vectors may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the polypeptide is produced as a fusion and/or nucleic acid encoding secretion signals so that the polypeptide  
15 produced in the host cell is secreted from the cell.

Vectors for production of polypeptides of the invention of for use in gene therapy include vectors which carry a mini-gene sequence of the invention.

20

For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid,  
25 for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

30

Vectors may be transformed into a suitable host cell as described above to provide for expression of a polypeptide of the invention. Thus, in a further aspect the invention provides a process for preparing polypeptides  
35 according to the invention which comprises cultivating a

host cell transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

5 Polypeptides may also be expressed in vitro systems, such as reticulocyte lysate.

A further embodiment of the invention provides host cells transformed or transfected with the vectors for the  
10 replication and expression of polynucleotides of the invention. The cells will be chosen to be compatible with the said vector and may for example be bacterial, yeast, insect or mammalian. The host cells may be cultured under conditions for expression of the gene, so that the encoded  
15 polypeptide is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium,  
20 as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers

25 Polynucleotides according to the invention may also be inserted into the vectors described above in an antisense orientation in order to provide for the production of antisense RNA or ribozymes.

30

#### Assays

The present invention also provides an assay for a compound capable of interacting with adenine binding GPCR  
35 polypeptides of the present invention, which assay

comprises: providing a G protein coupled receptor comprising at least one polypeptide according to the invention, contacting the receptor with a putative binding compound; and determining whether said compound is able to  
5 interact with said receptor.

In one embodiment of the assay, the receptor or subunits of the receptor may be employed in a binding assay. Binding assays may be competitive or non-competitive.  
10 Such an assay can accommodate the rapid screening of a large number of compounds to determine which compounds, if any, are capable of binding to the polypeptides. Subsequently, more detailed assays can be carried out with those compounds found to bind, to further determine  
15 whether such compounds act as agonists or antagonists of the polypeptides of the invention.

The present invention still further provides a bioassay for identifying compounds which modulate the activity of  
20 receptors comprising polypeptides of the invention. In one embodiment, the bioassay is conducted using *Saccharomyces cerevisiae* cells, engineered with GPCRs of the invention and rat or human G proteins to replace the endogenous GPCRs and G proteins, which normally function  
25 as the pheromone pathway required for conjugation and mating of the yeast cells. The normal yeast response to activating ligand (growth arrest) is also engineered to be converted to positive growth. With such engineering, the yeast cells can be used in functional assays to identify  
30 agonists and antagonists to the GPCRs of the invention, with growth indicating activation of the GPCR. For example, the bioassay may be conducted by contacting such yeast cells expressing the GPCR comprising a polypeptide of the invention with at least one potential agonist and  
35 thereafter monitoring the cells for changes in growth

activity. In yet another embodiment, the bioassay is conducted by contacting such yeast cells expressing receptor comprising a polypeptide of the invention with a constant amount of known agonist e.g. adenine and  
5 increasing amounts of at least one potential antagonist and thereafter monitoring the cells for changes in growth.

The present invention also provides a bioassay for identifying compounds which modulate the regulatory  
10 regions of the adenine binding GPCR gene. Such an assay is conducted utilising rat or human cells capable of expressing a polypeptide of the invention (preferably of SEQ ID NO:2 or SEQ ID NO:4). The cells are contacted with at least one compound wherein the ability of said compound  
15 to modulate the regulatory region is known. Thereafter, the cells are monitored for expression of the nucleic acid of the invention. Alternatively, the promoter may be linked to a reporter gene. Suitable reporter genes that may be employed include, for example, the chloramphenicol  
20 acetyltransferase gene, the luciferase gene, and the like.

A compound or a signal that "modulates the activity" of a polypeptide of the invention refers to a compound or a signal that alters the activity of the polypeptide so that  
25 it behaves differently in the presence of the compound or signal than in the absence of the compound or signal. Compounds affecting modulation include agonists and antagonists. An agonist encompasses a compound such as adenine which activates adenine binding GPCR function.  
30 Alternatively, an antagonist includes a compound that interferes with adenine binding GPCR function. Typically, the effect of an antagonist is observed as a blocking of agonist-induced receptor activation. Antagonists include competitive as well as non-competitive antagonists. A  
35 competitive antagonist (or competitive blocker) interacts



with or near the site specific for agonist binding. A non-competitive antagonist or blocker inactivates the function of the receptor by interacting with a site other than the agonist interaction site. As described below,  
5 guanine has a weak antagonistic effect at these receptors.

As understood by those of skill in the art, bioassay methods for identifying compounds that modulate the  
10 activity of receptors such as polypeptides of the invention generally require comparison to a control. One type of "control" is a cell or culture that is treated substantially the same as the test cell or test culture exposed to the compound, with the distinction that the  
15 "control" cell or culture is not exposed to the compound. Another type of "control" cell or culture that can be employed is a cell or culture that is identical to transfected cells, the exception that the "control" cell or culture does not express functional adenine binding GPCR.  
20 Accordingly, the response of the transfected cell can be compared with that of the "control" cell or culture to the same compound under the same reaction conditions.

Particularly preferred types of assays include binding  
25 assays and functional assays which may be performed as follows:

#### Binding assays

30 Over-expression of nucleic acid encoding polypeptides of the invention in cell lines (including mammalian HEK 293 cells, CHO cells and Sf9 insect cells) may be used to produce membrane preparations bearing said polypeptides (referred to in this section as adenine binding GPCR for  
35 convenience) for ligand binding studies. These membrane

preparations can be used in conventional filter-binding assays (eg. Using Brandel filter assay equipment) or in high throughput Scintillation Proximity type binding assays (SPA and Cytostar-T flashplate technology; Amersham Pharmacia Biotech) to detect binding of radio-labelled purinergic ligands (including [2-<sup>3</sup>H Adenine (Amersham, TRK311 and 8-<sup>3</sup>H Adenine (Amersham, TRK343] and displacement of such radio-ligands by competitors for the binding site. Radioactivity can be measured with Packard Topcount, or similar instrumentation, capable of making rapid measurements from 96-, 384-, 1536- microtitre well formats. SPA/Cytostar-T technology is particularly amenable to high throughput screening and therefore this technology is suitable to use as a screen for compounds able to displace standard ligands.

Another approach to study binding of ligands to adenine binding GPCR protein in an environment approximating the native situation makes use of a surface plasmon resonance effect exploited by the Biacore instrument (Biacore). Adenine binding GPCR in membrane preparations or whole cells could be attached to the biosensor chip of a Biacore and binding of ligands examined in the presence and absence of compounds to identify competitors of the binding site.

#### Functional assays

Since adenine binding GPCR acts via G<sub>i</sub> or G<sub>o</sub>(inhibitory G protein), which usually interacts with GIRK (inward rectifying potassium channels), potassium ion flux should result on activation of these receptors. This flux of ions may be measured in real time using a variety of techniques to determine the agonistic or antagonistic effects of particular compounds. Therefore, recombinant

adenine binding GPCR receptors expressed in cell lines or, for example, *Xenopus* oocytes can be characterised using whole cell and single channel electrophysiology to determine the mechanism of action of compounds of  
5 interest. Electrophysiological screening, for compounds active at adenine binding GPCR, may be performed using conventional electrophysiological techniques and when they become available, novel high throughput methods currently under development.

10

Fluorescence - Calcium and sodium fluxes are measurable using several ion-sensitive fluorescent dyes, including fluo-3, fluo-4, fluo-5N, fura red, Sodium Green, SBFI and other similar probes from suppliers including Molecular  
15 Probes. Calcium and sodium influx as a result of adenine binding GPCR can thus be characterised in real time, using fluorometric and fluorescence imaging techniques, including fluorescence microscopy with or without laser confocal methods combined with image analysis algorithms.

20

Another approach is a high throughput screening assay for compounds active as either agonists or modulators which affect calcium transients. This assay is based around an instrument called a **FLuorescence Imaging Plate Reader**  
25 ((FLIPR®), Molecular Devices Corporation). In its most common configuration, it excites and measures fluorescence emitted by fluorescein-based dyes. It uses an argon-ion laser to produce high power excitation at 488 nm of a fluorophore, a system of optics to rapidly scan the over  
30 the bottom of a 96-/384-well plate and a sensitive, cooled CCD camera to capture the emitted fluorescence. It also contains a 96-/384-well pipetting head allowing the instrument to deliver solutions of test agents into the wells of a 96-/384-well plate. The FLIPR assay is  
35 designed to measure fluorescence signals from populations

of cells before, during and after addition of compounds, in real time, from all 96-/384-wells simultaneously. The FLIPR assay may be used to screen for and characterise compounds functionally active at recombinant adenine binding GPCR, eg rat or human adenine binding GPCR, expressed in cell lines. As described below, calcium transients in cells transfected with adenine binding GPCR were measured using the FLIPR assay in order to measure activation of the receptors by various substrates in order to determine the natural ligand of the receptor.

Compounds found to modulate the activity of the polypeptides of the present invention have a number of therapeutic uses. As is obvious from the BLAST searches adenine binding GPCR does not belong to any of the known subfamilies of GPCRs that are activated structures similar to adenine. This suggests that adenine binding GPCR is a member of a new subgroup of purinergic GPCRs where it will be possible to develop a novel pharmacology for the different therapeutic areas in which purinergics have been applied up to now. In specific, this includes diseases related to apoptosis (Chow et al., 1997), pathological tremor disorders (Mally and Stone, 1996), nociception (Burnstock, 1997). Purinergic compounds have also been suggested for use as anticonvulsives, sedatives, hypnotics, ataxic (CNS), hypotensives, antiarrhythmics, negative chronotropics, dromotropics and inotropics, inhibitors of platelet aggregation, stimulators of NO production by vascular endothelial cells in the cardiovascular system and as inhibitors of gastric secretion (Gil-Rodrigo et al, Pharmacol. Res. 22(2):103-13, 1990). Furthermore studies based on transgenic animals suggest that receptors from this family play a role in bronchoconstriction, vasodilation, inflammation and facilitation of neurotransmission (Nyce, 1999). In

summary adenine binding GPCR and related receptors can be used as a valuable tool for drug development in a variety of therapeutic areas.

- 5 Furthermore the demonstration that adenine itself acts as the natural ligand to this receptor opens up the possibility of the use of adenine and compounds mimicking or antagonising adenine in the development of treatments for the diseases mentioned above.

10

#### Tissue distribution studies

- Adenine binding GPCR DNA sequences are useful for confirming and extending knowledge of this GPCR 's  
15 distribution by hybridisation/PCR studies in human health and disease. Recombinant adenine binding GPCR in cell lines are useful for characterising adenine binding GPCR function and a variety of biochemical and biophysical methods are available for assaying this receptor. Assays  
20 of the invention described herein are also useful for drug discovery screening purposes.

#### Binding agents

- 25 Thus the invention further provides novel binding agents, including modulatory agents obtained by an assay according to the present invention, and compositions comprising such agents. Agents which bind to the receptor and which may have agonist or antagonist activity may be used in methods  
30 of treating diseases whose pathology is characterised by action via the adenine binding GPCR receptor, and such use forms a further aspect of the invention. Such diseases may include diseases related to apoptosis, pathological tremor disorders and nociception bronchoconstriction,  
35 vasodilation, inflammation and facilitation of

neurotransmission. Furthermore, the agents may be used in treatments where anticonvulsives, sedatives, hypnotics, ataxic (CNS), hypotensives, antiarrhythmics, negative chronotropics, dromotropics and inotropics, inhibitors of platelet aggregation, stimulators of NO production by vascular endothelial cells in the cardiovascular system or as inhibitors of gastric secretion may be useful. The agents may be administered an effective amount of an agent of the invention. Since many of the above-mentioned conditions are chronic and often incurable, it will be understood that "treatment" is intended to include achieving a reduction in the symptoms for a period of time such as a few hours, days or weeks, and to include slowing the progression of the course of the disease.

Such agents may be formulated into compositions comprising an agent together with a pharmaceutically acceptable carrier or diluent. The agent may in the form of a physiologically functional derivative, such as an ester or a salt, such as an acid addition salt or basic metal salt, or an N or S oxide. Compositions may be formulated for any suitable route and means of administration. Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal, inhalable, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. The choice of carrier or diluent will of course depend on the proposed route of administration, which, may depend on the agent and its therapeutic purpose. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which

constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

For solid compositions, conventional non-toxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium carbonate, and the like may be used. The active compound as defined above may be formulated as suppositories using, for example, polyalkylene glycols, acetylated triglycerides and the like, as the carrier. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc, an active compound as defined above and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 15th Edition, 1975.

The composition or formulation to be administered will, in any event, contain a quantity of the active compound(s) in an amount effective to alleviate the symptoms of the

subject being treated.

Dosage forms or compositions containing active ingredient in the range of 0.25 to 95% with the balance made up from non-toxic carrier may be prepared.

For oral administration, a pharmaceutically acceptable non-toxic composition is formed by the incorporation of any of the normally employed excipients, such as, for example, pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, sodium crosscarmellose, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium carbonate, and the like. Such compositions take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained release formulations and the like. Such compositions may contain 1%-95% active ingredient, more preferably 2-50%, most preferably 5-8%.

Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, triethanolamine sodium acetate, etc.



The percentage of active compound contained in such parental compositions is highly dependent on the specific nature thereof, as well as the activity of the compound and the needs of the subject. However, percentages of active ingredient of 0.1% to 10% in solution are employable, and will be higher if the composition is a solid which will be subsequently diluted to the above percentages. Preferably, the composition will comprise 0.2-2% of the active agent in solution.

10

#### EXAMPLES

The following examples illustrate the invention. Other embodiments will occur to the person skilled in the art in light of these examples.

##### Example 1 Analysis of Novel GPCR

The sequence described in SEQ ID NO:1 was analysed by BLAST (Altschul et al., 1997) search to examine whether related GPCRs with known ligands could be found. However the closest homologues of this orphan GPCR that were retrieved, were orphan GPCRs themselves. These were a family of human GPCRs (Derwent sequence database accession numbers Z10067, Z10068, Z10069, Z10070 Z10071 and Z10072) cloned from dorsal root ganglion that have approximately 50% of residues in common with the novel GPCR (rat adenine binding GPCR). The next closest homologue is the mas related gene which shares 33% of residues with adenine binding GPCR.

Using the nucleotide sequence of SEQ ID NO:1, the present inventors have identified the human GPCRs above as being six human homologues of the rat novel GPCR. These have

35

been termed human adenine binding GPCR1 (SEQ ID NO:3), human adenine binding GPCR2 (SEQ ID NO:5), human adenine binding GPCR3 (SEQ ID NO:6), human adenine binding GPCR4 (SEQ ID NO:7), human adenine binding GPCR5 (SEQ ID NO:8) and human adenine binding GPCR6 (SEQ ID NO:9). The nucleotide and amino acid sequences for human adenine binding GPCR1 are shown in SEQ ID NO:3 and SEQ ID NO:4 with the amino acid sequences of rat adenine binding GPCR (SEQ ID NO:2) and human adenine binding GPCR.1 (SEQ ID NO:4) aligned in Figure 1. The nucleic acid sequence for human adenine binding GPCR2, human adenine binding GPCR3, human adenine binding GPCR4, human adenine binding GPCR5 and human adenine binding GPCR6 shown in SEQ ID NOs: 5-9 respectively. "N" in SEQ ID NO: 5 is A or T or C or G.

#### Example 2 Identification of the natural ligand of the novel GPCR

As no related GPCRs with known ligands could be found in the prior art, the inventors set about trying to identify which natural ligand would bind to the receptor. In order to identify the natural ligand of the sequenced GPCR, 'reverse pharmacology' was employed. Essentially an extract from the tissue where this GPCR is expressed (brain cortex) was prepared and the purification of the natural ligand was followed by a cellular assay based on the activation of the orphan GPCR.

#### Example 3 Transient expression in mammalian cells and FLIPR assay

The full length reading frame was inserted into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) and the resulting expression construct was transiently transfected with the FuGENE 6 reagent (Roche

Molecular Biochemicals, Mannheim, Germany) into HEK293 cells stably transfected (E. Bender, unpublished) with pLEC/Gqi or pLEC/Gqo (Molecular Devices, Sunnyvale, CA, U.S.A.). The cells were loaded with Fluo-4 (Molecular Probes, Eugene, OR, U.S.A.) according to the  
5 recommendations of the supplier.

#### Example 4 Preparation of tissue extracts and extract fractionation

10 10 kg porcine cortex were homogenized and extracted in methanol/water/acetic acid, 90/9/1, v/v/v. After centrifugation, the supernatant was delipidated by n-hexane extraction and the aqueous layer was fractionated  
15 by a Megabondelute fractionation. The material eluting at 50% acetonitrile/H<sub>2</sub>O was further fractionated on a HPLC C18 column. The fractions derived thereof were tested for activation of the novel GPCR in the FLIPR assay. Subsequent purification steps on an Hypersil C18, an  
20 analytical C8 column, a Biosep column and finally an HPLC C18 column were also followed by the FLIPR based activity assay for fractions activating the novel GPCR transfected cells. The fraction that contained the activity after the final purification step was analysed by mass spectrometry  
25 (Q-TOF, MicroMass, Manchester, U.K.).

The screening of the porcine cortex extract after the first fractionation on a C18 column delivered a rather broad range of fractions that resulted in transient  
30 intracellular Ca<sup>2+</sup> release for cells transiently transfected with the novel GPCR expression construct. This was the case for HEK293 cells expressing either the Gqo or the Gqi chimera (Figure 2 and Figure 3), but not for wild-type HEK293 cells (data not shown). One of these  
35 fractions, number 19 that yielded for both G protein

chimeras a strong stimulation was chosen for further subfractionation and processed as described above. The purification was guided by the FLIPR based activity assay and the fraction that showed activity from the last column run was subjected to mass spectrometry to determine purity as well as the structure of the compounds that it contained.

10 Example 5 Identification of activating substance by mass spectrometry

Mass analysis of the active purified fraction from the Symmetry C18 (4.6 x 250 mm; 5 $\mu$ m, Waters) reverse phase column gave one major ion at m/z 136. The mass was locked on the (NaI+Na)<sup>+</sup> ion (172.8840 Da) giving a more accurate mass for the ion of interest (136.0623 Da). This mass was used in the elemental composition analysis. The mass accuracy tolerance was set at 20 ppm. The best match was given by C<sub>5</sub>H<sub>6</sub>N<sub>5</sub> (136.0623 Da), corresponding to Adenine + H<sup>+</sup>. The CID spectrum of the native molecule was compared to that of synthetic adenine.

Fragmentation of both parent molecules resulted in the same fragment ions at m/z 119.0, 109.0, 94.0, 92.0, 82.0, 77.0, 67.0 and 65.0. The conclusion from the results obtained by tandem mass spectrometry on a Q-TOF was that the activating substance was adenine. The novel GPCR was therefore named adenine binding GPCR.

30 Example 6 Testing of novel GPCR with selected pure compounds

To confirm the result obtained from mass spectrometry pure adenine was dissolved in PBS to give final concentrations

3.3nM - 33 $\mu$ M and added to HEK293/Gaqo cells transiently transfected with the rat novel GPCR(adenine binding GPCR)/pcDNA3 expression construct and to those transfected with empty expression vector. Using the FLIPR assay as  
5 above, calcium transients were measured in both sets of cells with the transients attributable to the novel GPCR measured as the difference between the two transients. Results are illustrated in Figure 4. Adenine provoked a dose responsive calcium transient in the cells, indicating  
10 that the novel GPCR (adenine binding GPCR) has a high affinity for this compound. In contrast, other purines and pyrimidines did not demonstrate agonistic activity. Figure 5 illustrates that calcium transients could not be elicited in the HEK293/Gaqo cells by the addition of 3.3 $\mu$ M  
15 uridine, guanine, hypoxanthine, xanthine, caffeine, UDP or UDP, confirming the specificity of the adenine binding GPCR receptor for adenine. To further test the specificity of the receptor for adenine, a number of nucleoside and nucleotide derivatives were tested using  
20 the same assay. The results are shown in Figure 6. For the nucleoside and nucleotide derivatives of adenine only adenosine and AMP show some weak partial agonism at high concentrations (> 3.3  $\cdot$  M).

25 In order to examine whether among the same compounds, molecules with antagonistic properties could be found, cells transfected with adenine binding GPCR were exposed to various purines (guanine, hypoxanthine, xanthine, caffeine, theophylline), pyrimidines (uridine, UDP, UTP),  
30 known antagonists at other nucleotide receptors (suramin, reactive blue) and nucleoside or nucleotide derivatives of adenine (adenosine, AMP, ADP, ATP) at concentrations ranging from 30  $\mu$ M to 30 pM while measuring Ca<sup>2+</sup> transients in the FLIPR. No further agonistic effects

besides those mentioned above were discovered in this experiment. After a 15 minutes incubation at room temperature, the same cells were challenged with 30 nM adenine, to detect antagonistic properties of molecules added in the first round of superfusion. Only guanine showed a weak antagonistic effect at 30 and 3  $\mu$ M (Figure 7). All other compounds did not elicit antagonistic effects on adenine binding GPCR at the tested concentrations.

10

Examples 7 to 12 Characterization of the rat Adenine receptor on transiently and permanently transfected CHO cells

15 To further characterise the novel rat Adenine receptor, CHO cells were transiently and permanently transfected using the lipofectamine plus method. The pharmacological properties of a variety of adenine-derivatives were evaluated using radioligand binding experiments, stimulation of [ $^{35}$ S]GTP $\gamma$ S binding and cAMP measurements. All these experiments were run in parallel with wild type CHO cells as a control.

Example 7 describes radioligand binding experiments using [ $^3$ H] adenine in transiently and permanently transfected CHO cells. Measurement of binding of [ $^{35}$ S] GTP $\gamma$ S in response to adenine is described for transiently and permanently transfected cells in Examples 8 and 10 respectively. Measurement of cAMP in response to adenine is described for transiently and permanently transfected cells in examples 9 and 11 respectively. In example 12, the effect of adenine on intracellular Ca $^{2+}$  is described.

30

Cell Culture and Transfection Methods

CHO cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture Ham's F12 (Life Technologies, Belgium) in a ratio of 1:1 supplemented with  
5 10% heat inactivated fetal calf serum, and an antibiotic solution containing 100 IU/ml penicillin G, 100 µg/ml streptomycin sulfate, 110 µg/µl pyruvic acid and 300 µg/ml L-glutamine. The cells were grown in 175 cm<sup>2</sup> culture flasks at 37°C in a 5% CO<sub>2</sub> environment. For the  
10 permanently transfected cells, once every two weeks, the transfected cells were cultured in a selection medium (Dulbecco's modified Eagle's medium (DMEM) nutrient mixture Ham's F12 (ratio 1:1), Life Technologies, Belgium) containing 800 µg/ml geneticine (G418).

15 For transient transfections of CHO cells the lipofectamine PLUS (Life Technologies) method was used. One day before the transfection, the CHO cells were seeded out in 50 mm petri dishes at a density of 20,000 cells/cm<sup>2</sup> in DMEM  
20 nutrient mixture Ham's F12 (ratio 1:1) (Life Technologies). For the transfection of one petri dish, 6.5 µg DNA was added to 17.5 µl PLUS reagent (Life Technologies), mixed and incubated for 15 minutes at room temperature (solution A). 15 µl of lipofectamine PLUS  
25 reagent was added to 735 µl serum free medium (solution B). Solution A and B were combined, mixed gently and incubated for 15 minutes at room temperature to form a complex. After 15 minutes, 3.5 ml serum-free medium was added to the mix. The cells were washed once with serum-  
30 free medium and the DNA-lipofectamine PLUS complex (in a final volume of 5 ml) was added onto the cells and incubated for 3 hours at 37°C in a 5% CO<sub>2</sub> incubator. After the incubation, 5 ml of DMEM/Ham's F12 (ratio 1:1), supplemented with 20% heat inactivated fetal calf serum,

was added onto the cells and left overnight in the incubator. The next day, the medium was removed and replaced with the same growth medium as above.

- 5 The lipofectamine PLUS method was also used for permanent transfection of CHO cells with slight modifications. One day after the transfection, the medium was replaced with selection medium which was replaced every 2 to 3 days. The cells were grown till colonies appeared (between day 8  
10 and 10) and split to a 175 cm<sup>2</sup> culture flask for further culture and experiments. Monoclonal cell lines were prepared by limited dilution seeding in 96 well plates.

#### Membrane preparation

15

- The membranes were prepared as total particulate fractions. The cell lines were cultured to 90% confluency on 145 mm petri dishes and treated with 5 mM sodium butyrate, 24 hours before collection. The culturing  
20 medium was removed and the cells were washed twice with ice cold phosphate buffered saline (PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>), scraped from the plates in 50 mM Tris-HCl buffer, pH 7.4, and collected by centrifugation (10 minutes at 16,000 RPM at 4°C). The cell pellet was re-suspended in  
25 hypotonic 5 mM Tris-HCl buffer, pH 7.4, and homogenized with an Ultra Turrax homogenizer (Janker Kunkel, Germany). The homogenate was centrifuged at 18,000 RPM for 20 minutes at 4°C. The final pellet was re-suspended in 50 mM Tris-HCl buffer, pH 7.4 and stored in aliquots at -  
30 70°C. A protein determination was performed using the Bradford protein assay (Biorad, Germany) using bovine serum albumin (BSA) as standard.

#### Example 7 [<sup>3</sup>H]Adenine binding

35



A) Methods - [ $^3\text{H}$ ]Adenine binding experiments were performed to characterize wild type cell lines before transfection as well as transiently and permanently transfected cell lines. Membranes were thawed on ice and  
5 diluted in 50 mM HEPES buffer, pH 7.4 supplemented with 10 mM  $\text{MgCl}_2$  and 1 mM EGTA. Non-specific binding was defined in the presence of 1  $\mu\text{M}$  adenine and a 1 hour incubation at 25°C with [ $^3\text{H}$ ]adenine (specific activity of 30.4 Ci/mmol), was found to be optimal for saturation and competition  
10 binding assays. Both assays were carried out in a final volume of 500  $\mu\text{l}$ , using 18  $\mu\text{g}$  of membrane protein for the transfected CHO cells and 30  $\mu\text{g}$  of membrane protein for the wild type CHO cells for the saturation binding. For competition binding experiments, 15 and 50  $\mu\text{g}$  of membrane  
15 protein was used for the transfected and wild type cells, respectively. The reaction was terminated by rapid filtration through Whatman GF/B filters using a Brandel multi-channel cell harvester (96 wells). The filters were washed three times with 3 ml ice cold 50 mM HEPES buffer, pH 7.4, transferred to liquid scintillation vials and 3 ml  
20 of scintillation fluid (Ultima Gold MV, Packard, Netherlands) was added. Samples were counted in a  $\beta$ -scintillation counter after at least 6 hours to permit the glass fiber filters to become uniformly translucent.  
25

Saturation analysis of [ $^3\text{H}$ ]adenine binding was carried out using 18 concentrations of the radioligand ranging from 0.2 to 90 nM.

30 Competitive inhibition of [ $^3\text{H}$ ]adenine by the compounds, adenine, 1-methyladenine and other analogues was performed with 15 nM of [ $^3\text{H}$ ]adenine and various concentrations of the unlabeled compounds, spanning 5 orders of a magnitude.

35 The maximum number of binding sites ( $B_{\text{max}}$ ), the apparent

equilibrium dissociation constant ( $K_D$ ) and the  $pIC_{50}$  values (as  $IC_{50}$  value represents the concentration that inhibits 50% of specific radioligand binding) were derived from non-linear curve fitting.

5

b) Results: Preliminary radioligand binding experiments with [ $^3H$ ]adenine were conducted on different wild-type cells. The assay conditions were identical to those for the transfected cells. Binding results obtained with the different cell lines are shown below:

10

C6 glioma:	839 fmoles/mg protein
Cos7:	226 fmoles/mg protein
HEK293:	168 fmoles/mg protein
15 NIH3T3:	642 fmoles/mg protein
CHO:	303 fmoles/mg protein (pool 1)
	583 fmoles/mg protein (pool 2)

The CHO cells were chosen for transfection with the rat adenine receptor and further experiments.

20

In independent experiments 48 hours post transfection (efficiency was 50-60 % as evaluated by  $\beta$ -gal staining using conventional techniques known to the skilled person) CHO cells were evaluated for [ $^3H$ ]adenine binding. In preliminary binding experiments performed using 4 nM [ $^3H$ ]adenine, the following binding levels were observed:

25

pool 1	3554 fmoles/mg protein
30 pool 2	3444 fmoles/mg protein
pool 3	2436 fmoles/mg protein
pool 4	1717 fmoles/mg protein

Saturation binding analysis of [ $^3H$ ]adenine, was carried out with and without addition of 100  $\mu$ M

35

guanylylimidodiphosphate (Gpp-NHp) on the transfected as well as on the wild type CHO cells, using an average of 16 radioligand concentrations ranging from 0.1 to 80 nM (table 1). Addition of Gpp-NHp blocks the high-affinity or agonist binding sites of GPCRs, so that only low-affinity sites are available.

In transfected cells average saturation binding levels were around 19 pmol/mg and the  $K_D$ -value for [ $^3$ H]adenine was 24 nM. Specific binding with similar  $K_D$  was also observed in wild type CHO cells, but with a much lower  $B_{max}$  (~ 1.1 pmol/mg). Furthermore, the  $K_D$  of transfected cells was sensitive to Gpp-NHp addition, whereas the  $K_D$  in wild type cells was not.

Competitive inhibition of [ $^3$ H]adenine binding by a variety of adenine derivatives was performed using 15 nM of [ $^3$ H]adenine on cells which had been transiently transfected with the receptor and on the wild type CHO cells (table 2).

High affinity competition binding could only be obtained with adenine itself, with a  $K_i$ -value of 18 nM, which is very close to the directly measured  $K_D$ -value.

#### Example 8 [ $^{35}$ S]GTP.S binding in transiently infected cells

a) Methods - Determination of [ $^{35}$ S]GTP.S binding to the G-proteins was carried out according to a modified procedure of Lazareno (Methods Mol. Biol., 83:107-116, 1997). These experiments were performed for a first functional evaluation of the receptor on the level of a membrane preparation.

In preliminary [ $^{35}$ S]GTP.S binding experiments, assay

conditions were optimized, which resulted in the choice of the following incubation buffer: 20 mM Hepes, pH 7.4 containing 100 mM NaCl, 10  $\mu$ M GDP, 10 mM MgCl<sub>2</sub> and 10  $\mu$ g/ml saponine. The assay mixture contained 10 and 35  $\mu$ g of membrane protein for the transfected and wild type CHO cells, respectively.

For testing agonistic activity, 175  $\mu$ l of diluted membranes was pre-incubated in the buffer described above together with 25  $\mu$ l of buffer and 25  $\mu$ l of varying concentrations of the compound in a total volume of 225  $\mu$ l. After a 20 minute pre-incubation period at 30°C, 25  $\mu$ l of [<sup>35</sup>S]GTP $\gamma$ S was added to a final concentration of 0.25 nM and the assay mixtures were further incubated for 20 minutes at 30°C. Bound and free [<sup>35</sup>S]GTP $\gamma$ S were separated by rapid filtration over an UniFilter<sup>TM</sup>-96, GF/B<sup>TM</sup> under reduced pressure using Filtermate 196 filtration (Packard Company (Netherlands)). The amount of bound radioactivity on the filter unit was determined after drying of the filters and scintillant addition (Microscint-O; Packard Company) by liquid scintillation counting.

Basal [<sup>35</sup>S]GTP $\gamma$ S binding was measured in absence of compounds. Stimulation by agonist was calculated as the percentage increase above basal levels. The sigmoid agonist concentration-response curves were analyzed by non-linear regression using the GraphPad Prism program. The different concentration points were run in duplicates and the reported data were retrieved from independent experiments.

b) Results - [<sup>35</sup>S]GTP $\gamma$ S binding on membranes from transiently transfected CHO cells had a maximum

stimulation by adenine of 150% above basal binding levels.

The mean EC<sub>50</sub> for adenine was 112 nM. 1-methyladenine and AMP stimulated the receptor partially with a much lower potency, in correspondence with their lower affinity in competition binding assays.

No stimulation of GTPγS binding was measured in wild type CHO cells (see table 3 and Figure 8a (transiently transfected CHO) and Figure 8b(wild type CHO)). The values in percentages are normalized to maximum adenine response, i.e. the adenine stimulation over basal levels is taken as 100%.

Example 9 cAMP measurements in transiently transfected CHO cells

a) Methods - For transiently transfected CHO cells, cAMP measurements were performed using the direct RIA kit from NEN (Belgium).

The cells were collected from 50 mm petri dishes by adding 1 ml 0.04% EDTA and re-suspending them in PBS (without Ca<sup>2+</sup> and Mg<sup>2+</sup>). The cells were then centrifuged for 5 minutes at 1,500 RPM, the supernatant was removed and the pellet was re-suspended in stimulation buffer (provided by the supplier, NEN). The cells were counted and 50 μl was added to each well of a 96-well flashplate at a density of 10<sup>6</sup> cells/well. A standard curve of cAMP was prepared in stimulation buffer, covering a range from 10 to 1,000 nM, and 50 μl was added to the flashplate.

The compounds were diluted in PBS with forskolin (at 50 μM), and 50 μl of the ligand, 2 times the final concentration, was added to the cells and incubated for 20 minutes at 37 °C. After the 20 minutes incubation, 100 μl

of the [ $^{125}$ I]cAMP tracer (2  $\mu$ Ci) was added to each well, resulting in a final volume of 200  $\mu$ l/well. The plate was left at room temperature, until being counted in the Topcount<sup>TM</sup> microplate scintillation counter (Packard, Netherlands). The sigmoid concentration response curves (duplicate points) were analyzed using the GraphPad Prism program (GraphPad Software, CA, USA).

b) Results - Inhibition of forskolin stimulated cAMP with the direct method was very low (15-30 %). Forskolin, basal and pEC<sub>50</sub> values of both methods are given in table 4; representative curves from the assay are shown in Figure 9a (0.5%hiFCS) and Figure 9b(2%hiFCS).

Although the response window of the assays was low, the assay confirmed the agonistic activity of adenine on the receptor and the measured EC<sub>50</sub> (~15 nM) were close to its adenine affinity

Since the low signal in the functional cAMP assay on transiently transfected cells could be due to the heterogeneous nature (transfected and non-transfected cells) of the cells it was decided to select permanently transfected cell lines.

A number of monoclonal cell lines were investigated by radioligand binding as described above with three (clone 26 (mother plate 1), 28, 29 and 30 (mother plate 9) chosen for further studies.

Example 10 [ $^{35}$ S]GTP $\gamma$ S binding in permanently transfected cells

The first functional characterization of the monoclonal

cell lines was performed in [<sup>35</sup>S]GTPγS binding experiments.

The maximum stimulation of [<sup>35</sup>S] GTPγS binding by adenine for each of the 4 selected clones was much higher (500-650-fold) than the that found in the transient

5 transfections confirming the expected better coupling in permanent cell lines (see table 5). The mean EC50 of 62 nM, is however close to the transient preparation (cf Example 8). An example of [<sup>35</sup>S]GTPγS binding stimulation by adenine is given in Figure 10.

10

Example 11 cAMP measurements in permanently transfected CHO cells

For permanently transfected CHO cells, cAMP measurements  
15 were performed using the indirect RIA kit from NEN (Belgium).

a) Methods: Two days before the experiment the cells were seeded out in a 96 well plate at a density required for  
20 approximately 90% confluence at the day of the experiment.

After removal of the growth medium, the cells were washed twice with a controlled salt solution (CSS) containing 25 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 15 mM glucose and 15 mg/l phenol red,  
25 with an addition of a phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine) in a concentration of 1 mM and 0.1% BSA. Basal cAMP levels were determined in CSS containing these additives and maximally stimulated cAMP levels were determined in the presence of 50 μM forskolin.

30 For testing agonistic activity, the compounds were incubated with the cells for 20 minutes at 37 °C. The reaction was stopped by addition of ice cold HClO<sub>4</sub> (1N) and stored at -20°C. After thawing the mixtures were neutralized with an equivalent amount of phosphate

buffered KOH, left at 4°C during 30 minutes to allow the salts to precipitate and centrifuged at 2,000 RPM for 5 minutes at 4°C.

- 5 For the quantitative determination of the cAMP levels, a 96-well Flashplate radioimmunoassay kit from NEN was used, according to the protocol of the supplier. The sigmoid concentration response curves (duplicate points) were analyzed using the GraphPad Prism program.

10

b) Results - The effects of Na-butyrate induction and different serum concentrations were evaluated.

- Investigating the 4 selected clones, clone 28 (MP9) showed highest (-75 %) inhibition of forskolin stimulated cAMP  
15 levels. Wild type CHO cells did not show any response to adenine. Forskolin, basal and pEC<sub>50</sub> values of clone 28 and wild type CHO cells are given in table 6. A dependable high window in the cAMP experiments was only obtained after Na-butyrate induction of receptor expression levels.  
20 The measured EC<sub>50</sub> was in the low nanomolar range (3 nM). Representative concentration responses curves are given in Figure 11a and Figure 11b for permanently transfected and wild-type CHO cells respectively.

25 Example 12 Intracellular calcium measurements in permanently transfected CHO cells

Finally, measurements of intracellular Ca<sup>2+</sup> were performed on the best functionally coupled CHO cell line, clone 28.

- 30 The effect of Na-butyrate pre-treatment was investigated using a dose response curve of adenine.

- a) Methods - Two days before the experiment the cells were seeded out in a 96 well plate at a density required for  
35 approximately 90% cell confluence at the day of the



experiment. Compounds were diluted in calcium buffer (5 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 1 mM MgCl<sub>2</sub>, 5 mM KCl, 10 mM glucose, 1.25 mM CaCl<sub>2</sub>, 2.5 mM probenecid, pH 7.4), twice their final concentrations. For basal values, calcium buffer was applied and for the maximum response, 10  $\mu$ M ionomycin was used. The growth medium was removed and the cells were loaded in serum-free growth medium supplemented with probenecid (5 mM), the indicator Fluo-3 (2  $\mu$ M), 20 mM HEPES/NaOH, pH, 7.4 and 0.1% BSA in the incubator at 37°C. After 60 minutes incubation, the cells were washed 3 times with the calcium buffer. 100  $\mu$ l of the diluted compounds was transferred to the wells and changes in intracellular calcium levels were measured with the FLIPR<sup>®</sup> (Fluorometric Imaging Plate Reader, Molecular Devices). Each data point was run in duplicate and the peak of the Ca<sup>2+</sup>-transient was considered as the relevant signal. The sigmoid concentration response curves were analyzed using the GraphPad Prism program.

b) Results - Wild type CHO cells did not show any response to adenine. For non-Na-butyrate treated cells, adenine caused a 1.25-fold increase of the basal relative fluorescence units (RFU) ranging from 6800 to 8500. For the Na-butyrate treated cells, adenine induced a 3-fold increase in RFU ranging from 6600 to 19800. The pEC<sub>50</sub> values of the adenine response were 7.14 and 7.83, respectively. A representative example of the Ca<sup>2+</sup> response triggered by adenine is given in Figure 12a and Figure 12b for permanently transfected and wild-type CHO cells respectively.

Table 1 [<sup>3</sup>H]adenine saturation binding on CHO membranes,  
transiently transfected with adenine binding rat receptor vs.  
wild type CHO cells

Conditions	Transfected cells						
	<i>B</i> <sub>max</sub> (fmoles/mg protein)	<i>sd</i>	<i>sem</i>	<i>K</i> <sub>d</sub> (nM)	<i>sd</i>	<i>sem</i>	<i>n</i>
No Gpp-NHp	17569			24			
	13738			25			
	25375			23			
	18894	5931	3424	24	1	0.6	3
100 μM Gpp-NHp	12934			57			
	31236			72			
	7852			54			
	17341	12299	7101	61	10	7	3

5

Conditions	Wild type cells						
	<i>B</i> <sub>max</sub> (fmoles/mg protein)	<i>sd</i>	<i>sem</i>	<i>K</i> <sub>d</sub> (nM)	<i>sd</i>	<i>sem</i>	<i>n</i>
No Gpp-NHp	1901			34			
	1168			15			
	362			30			
	1144	770	444	26	10	6	3
100 μM Gpp-NHp	516			5			
	797			25			
	257			17			
	523	270	156	16	10	6	3

Table 2: [<sup>3</sup>H]adenine competition binding on CHO membranes,  
transiently transfected with adenine binding rat receptor vs.  
wild type CHO cells

Compound listing	Transfected cells				Wild type cells			
	<i>pIC<sub>50</sub></i>	<i>sd</i>	<i>K<sub>i</sub> (nM)</i>	<i>n</i>	<i>pIC<sub>50</sub></i>	<i>sd</i>	<i>K<sub>i</sub> (nM)</i>	<i>n</i>
Adenine	7.51		19		7.13		46	
	7.48		20		6.8			
	7.76		11		6.67		134	
	7.44		22					
	7.55	0.14	18	4	6.87	0.24	85	3
1-Methyladenine	5.21		3791		< 4			
	5.01		6056		< 4			
	5.24		3573					
	5.15	0.13	4391	3	< 4			2
AMP	4.29		31887		< 4			
	4.47		20847					
	4.46		21210					
	4.41	0.10	24503	3	< 4			1
ADP	< 4				< 4			
	< 4							
	< 4			2	< 4			1
ATP	4.02		58502		< 4			
	4.18		40730					
	4.02		58500					
	4.07	0.09	52789	3	< 4			1
Adenosine	< 4				< 4			
	4.14		44554					
	4			2	< 4			1
6-Benzylaminopurine	4.00		61401		< 4			
	4.06		54125					
	4.03	0.04	58328	2	< 4			1
Hypoxanthine	< 4				< 4			
	< 4							
	< 4			2	< 4			1
Guanine	< 4				< 4			
	< 4							
	< 4			2	< 4			1
Uridine	< 4				< 4			
	< 4							
	< 4			2	< 4			1
Adrianamycin	4.35		27792		< 4			
	4.35		27918	1	< 4			1
Daunomycin	4.30		30990		< 4			
	4.30		31324	1	< 4			1

Table 3: [<sup>35</sup>S]GTPγS binding on CHO membranes, transiently transfected with adenine binding rat receptor vs. wild type CHO cells

Compound listing	Transiently transfected cells						
	<i>pEC<sub>50</sub></i>	<i>sd</i>	<i>sem</i>	% stim.	<i>sd</i>	<i>sem</i>	<i>n</i>
Adenine	6.88			106			
	6.94			111	=100% ref		
	7.02			177	=100% ref		
	6.94			199	=100% ref		
	6.95	0.06	0.03	148	47	23	4
1-Methyladenine	4.79			90	(81% ref)		
	4.92			135	(76% ref)		
	4.75			161	(81% ref)		
	4.82	0.09	0.05	129	36	21	3
AMP	4.12			55	(50% ref)		
	4.56			92	(52% ref)		
	4.38			137	(69% ref)		
	4.35	0.22	0.13	95	41	24	3
6-Benzylaminopurine	< 4			12	(11% ref)		
	< 4			12			1

5

Compound listing	Wild type cells						
	<i>pEC<sub>50</sub></i>	<i>sd</i>	<i>sem</i>	% stim.	<i>sd</i>	<i>sem</i>	<i>n</i>
Adenine	< 5			0			
	< 5			0			
	< 5			0			
				0			3
1-Methyladenine	< 4			0			
	< 4			0			
				0			2
AMP	< 4			0			
	< 4			0			
				0			2
6-Benzylaminopurine							

**Table 4: cAMP measurements on CHO cells, transiently transfected with adenine binding rat receptor**

	<i>basal</i> (pmol/well)	<i>forskolin 50 <math>\mu</math>M</i> (pmol/well)	<i>pEC<sub>50</sub></i> <i>adenine</i>	<i>pEC<sub>50</sub></i> <i>1-methyl</i> <i>adenine</i>
Direct assay				
0.5% hiFCS	2.8	145	7.68	5.31
2% hiFCS	2.5	105	8.15	5.20
Indirect assay				
0.5% hiFCS	0.5	74	8.48	5.10
2% hiFCS	0.4	62	6.96	no fit

**Table 5: [<sup>35</sup>S]GTP $\gamma$ S binding on CHO membranes, permanently transfected with adenine binding rat receptor. Stimulation above basal by adenine**

	Permanently transfected cells	
	<i>pEC<sub>50</sub></i>	% stimulation
Clone 26 (MP1)	7.19	632
Clone 28 (MP9)	7.31	657
Clone 29 (MP9)	7.10	527
Clone 30 (MP9)	7.24	534

5

**Table 6: cAMP measurements on CHO cells, permanently transfected with adenine binding rat receptor**

	<i>Basal</i> (pmol/well)	<i>Forskolin 50 <math>\mu</math>M</i> (pmol/well)	<i>pEC<sub>50</sub></i> <i>adenine</i>
wild type			
0.5% hiFCS, no induction	0.27	26	/
0.5% hiFCS, induction	0.59	25	/
10% hiFCS, no induction	0.64	107	/
10% hiFCS, induction	1.08	63	/
transfected cells			
0.5% hiFCS, no induction	0.39	44	/
0.5% hiFCS, induction	0.71	45	8.54
10% hiFCS, no induction	0.68	142	/
10% hiFCS, induction	1.24	65	8.53

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CLAIMS

1. An isolated nucleic acid sequence encoding the polypeptide of SEQ ID NO:2 or SEQ ID NO:4.
2. An isolated nucleic acid sequence comprising SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 SEQ ID NO:7, SEQ ID NO:8 or SEQ ID NO:9.
3. An isolated nucleic acid sequence having at least 50% sequence identity to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 SEQ ID NO:7, SEQ ID NO:8 or SEQ ID NO:9.
4. An isolated polypeptide having the sequence of SEQ ID NO:2 or SEQ ID NO:4.
5. An isolated polypeptide having at least 50% sequence identity to the polypeptide of SEQ ID NO:2 or SEQ ID NO:4.
6. An isolated polypeptide according to claim 5 wherein the polypeptide retains adenine binding activity.
7. An isolated polypeptide comprising a fragment of the polypeptide of claim 4 or 5 and retaining adenine binding activity.
8. An isolated nucleic acid encoding a polypeptide as defined in claim 4, 5, 6, or 7.
9. An expression vector comprising a nucleic acid as defined in any one of claims 1-3 or claim 8 operably linked to a promoter.



10. A host cell carrying a vector according to claim 9.
11. A nucleic acid primer consisting essentially of a fragment of from 15 to 50 nucleotides of a nucleic acid encoding a polypeptide according to any one of claims 4, 5, 6, or 7.
12. An assay for a compound capable of interacting with a G protein coupled receptor, which assay comprises:
  - providing a G protein coupled receptor comprising at least one polypeptide according to any one of claims 4, 5, 6, or 7;
  - contacting said receptor with a putative interacting compound; and
  - determining whether said compound is able to interact with said receptor.
13. An assay according to claim 12, wherein said interaction is determined by the ability of the compound to bind to said receptor.
14. An assay according to claim 12, wherein said interaction is determined by the ability of the compound to modulate the activity of said receptor.
15. An assay according to any one of claims 12 to 14 wherein said receptor is located in the membrane of a cell in which the polypeptide according to any one of claims 4, 5, 6, or 7 is expressed from a vector according to claim 9.
16. Use of an assay according to any one of claims 12 to 15 for the identification of a modulator compound for the treatment of a disease, the pathology of which is associated with action at purine receptors.

17. A novel compound obtained by an assay according to any one of claims 12 to 15.

18. Use of a compound according to claim 17 in the preparation of a medicament for the treatment of a disease, the pathology of which is associated with action at purine receptors.

19. Adenine for use in a method of treatment in a human or animal body.

20. Use of adenine in the preparation of a medicament for the treatment of a disease, the pathology of which is associated with action at purine receptors.

21. An isolated polypeptide according to any one of claims 4, 5, 6, or 7 for use in a method of treatment in a human or animal body.

22. Use of an isolated polypeptide according to any one of claims 4, 5, 6, or 7 in the preparation of a medicament for the treatment of a disease, the pathology of which is associated with action at purine receptors.

23. Use of an isolated nucleic acid according to any one of claims 1, 2, 3 or 8 in the preparation of a medicament for the treatment of a disease, the pathology of which is associated with action at purine receptors.

Figure 1

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Figure 2

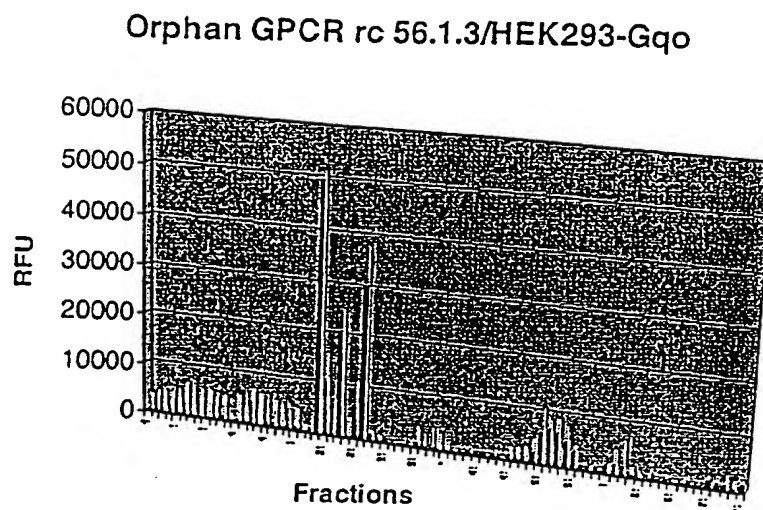


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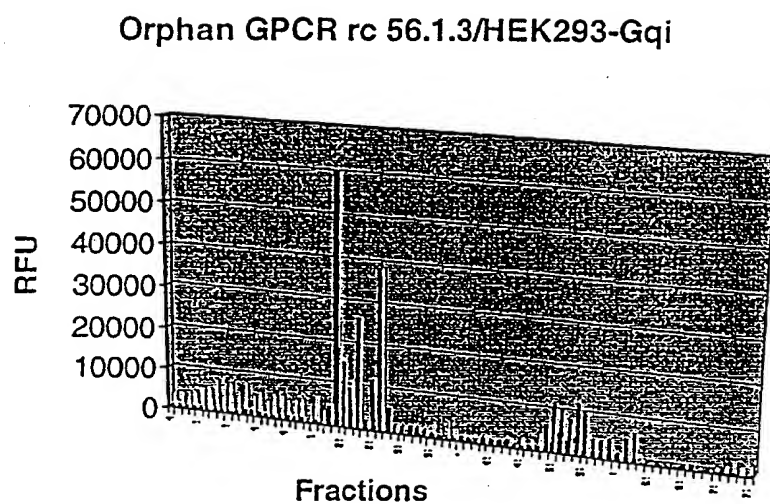


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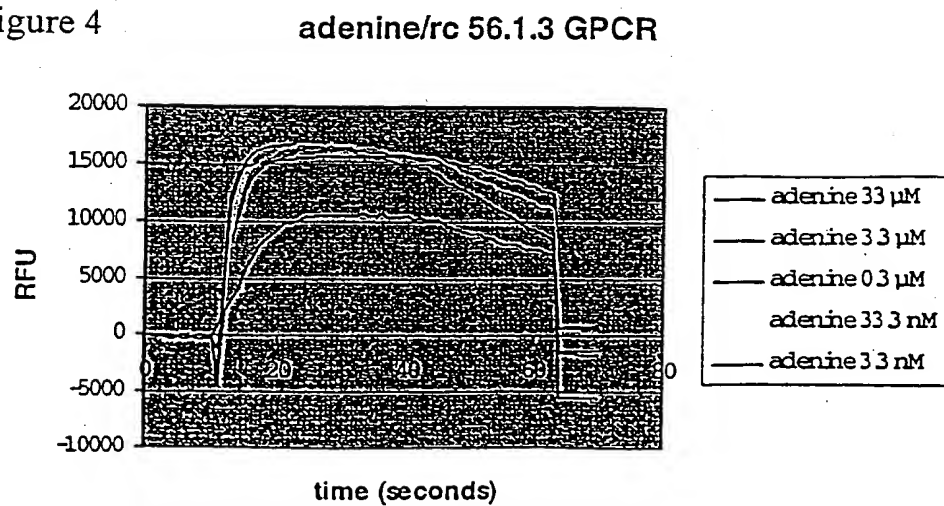


Figure 5

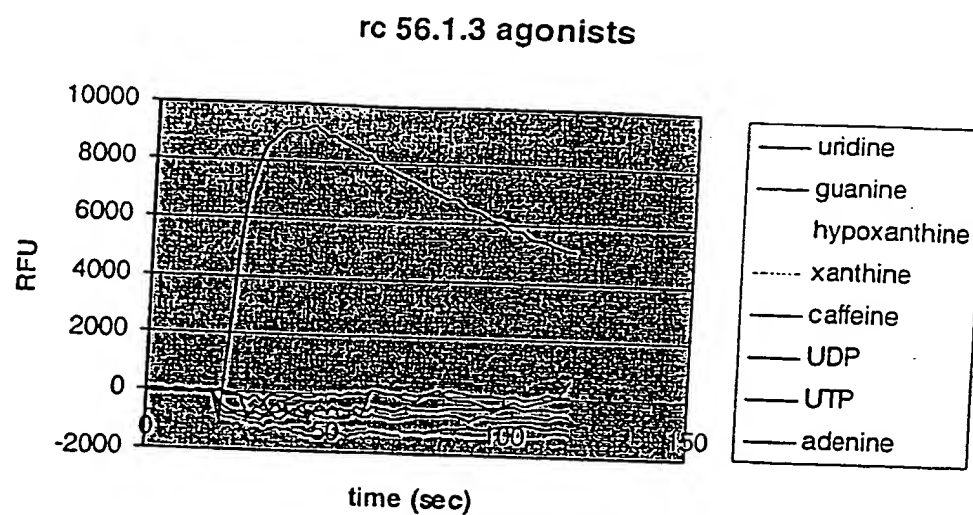


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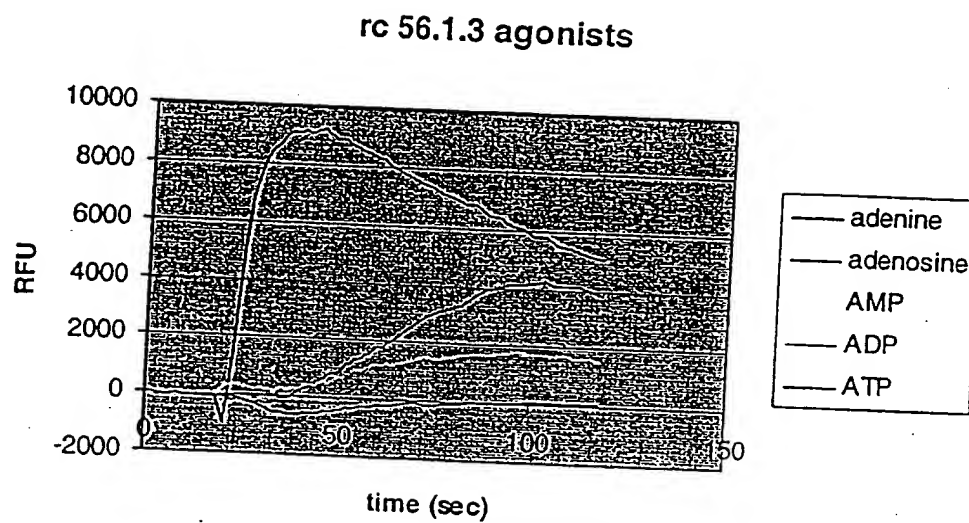


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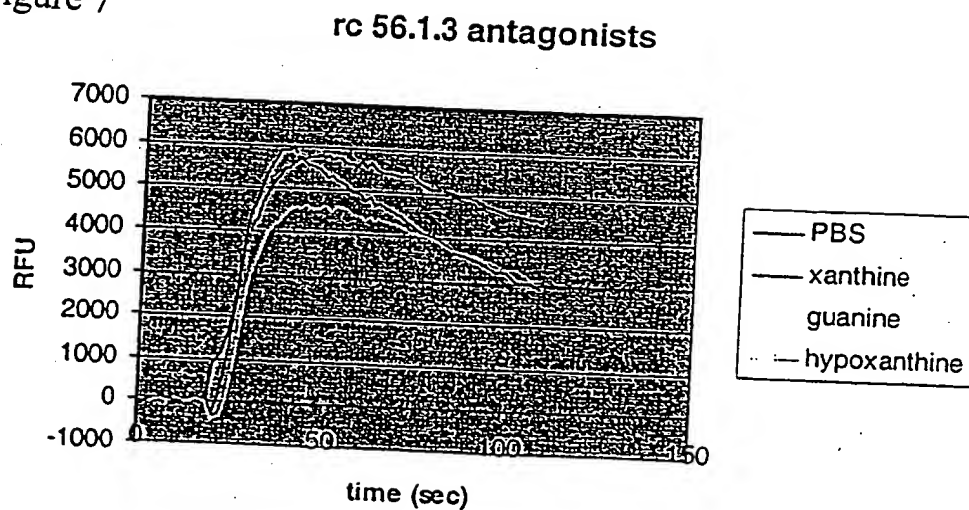


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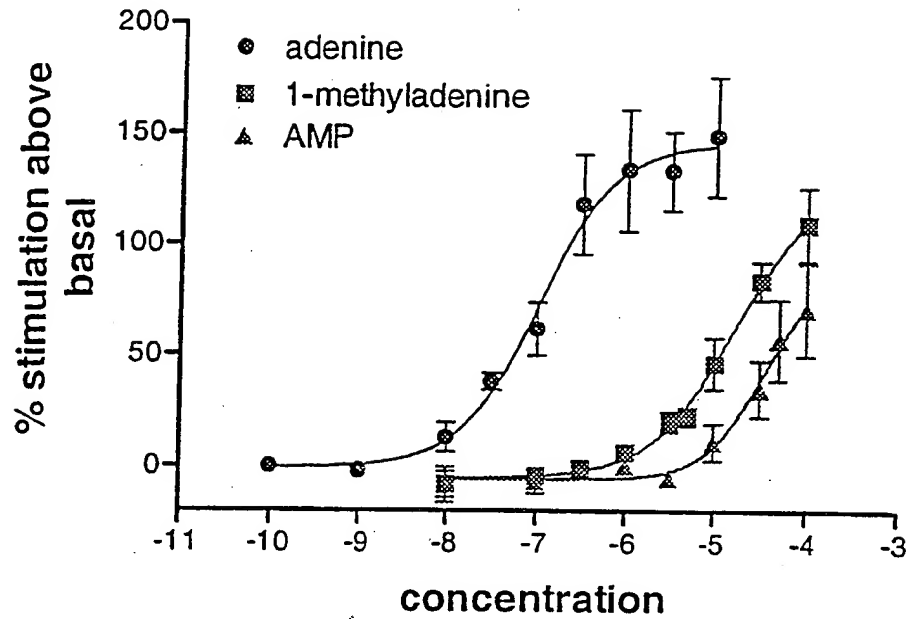


Figure 8b

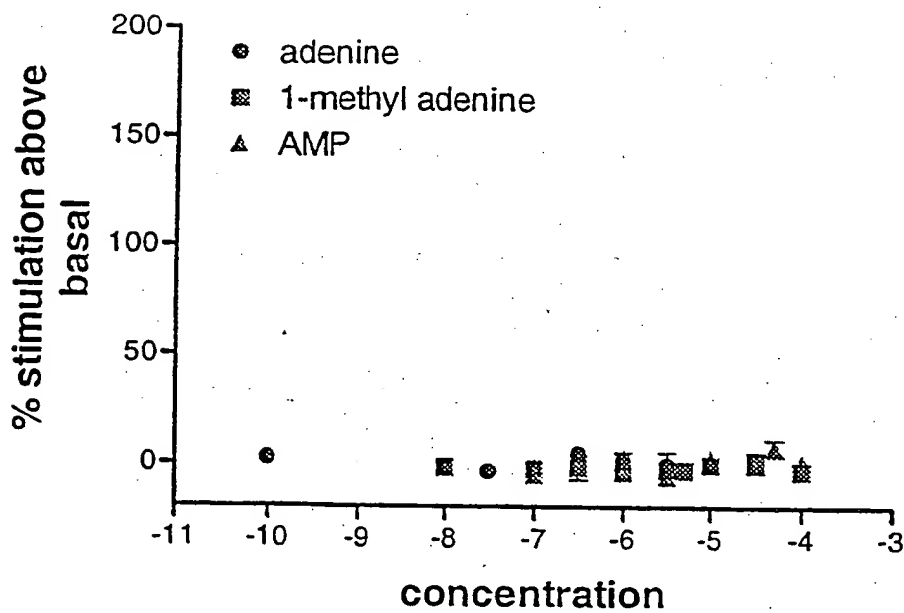


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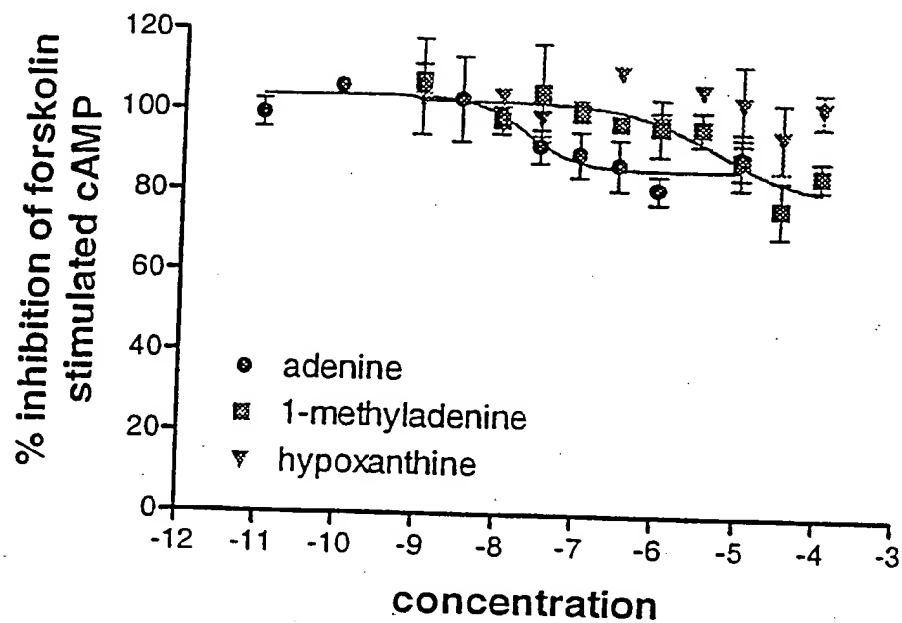


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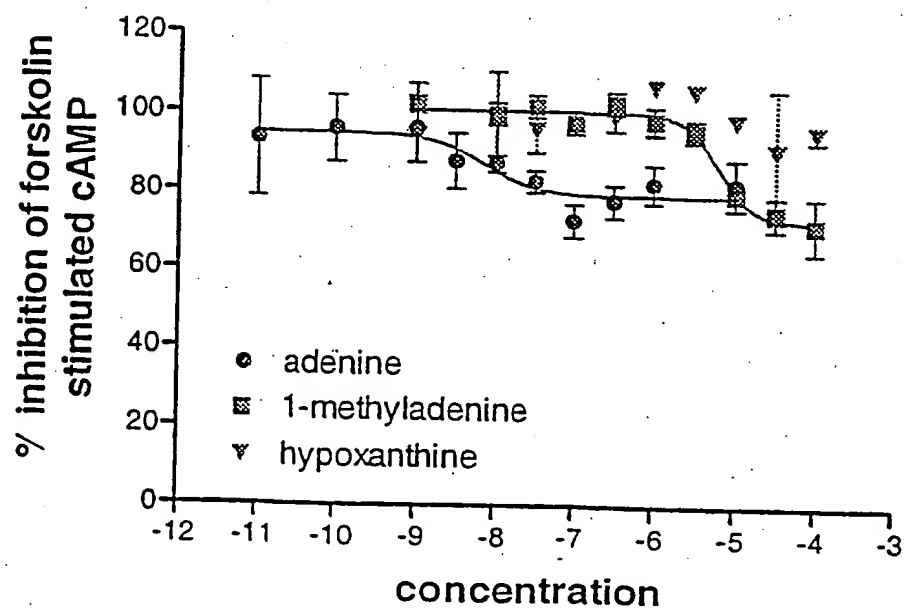


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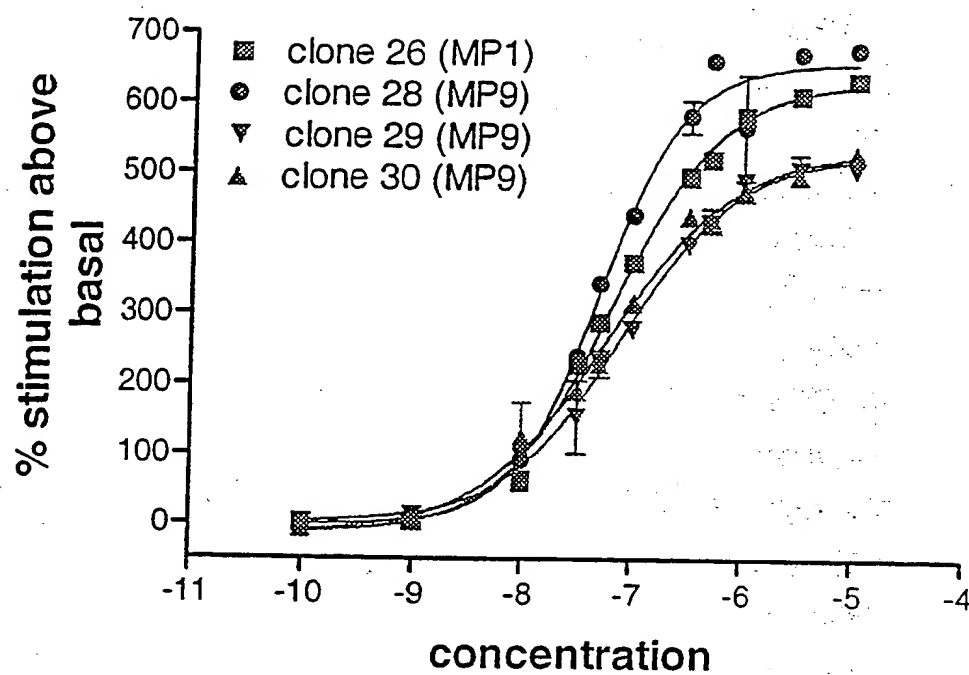




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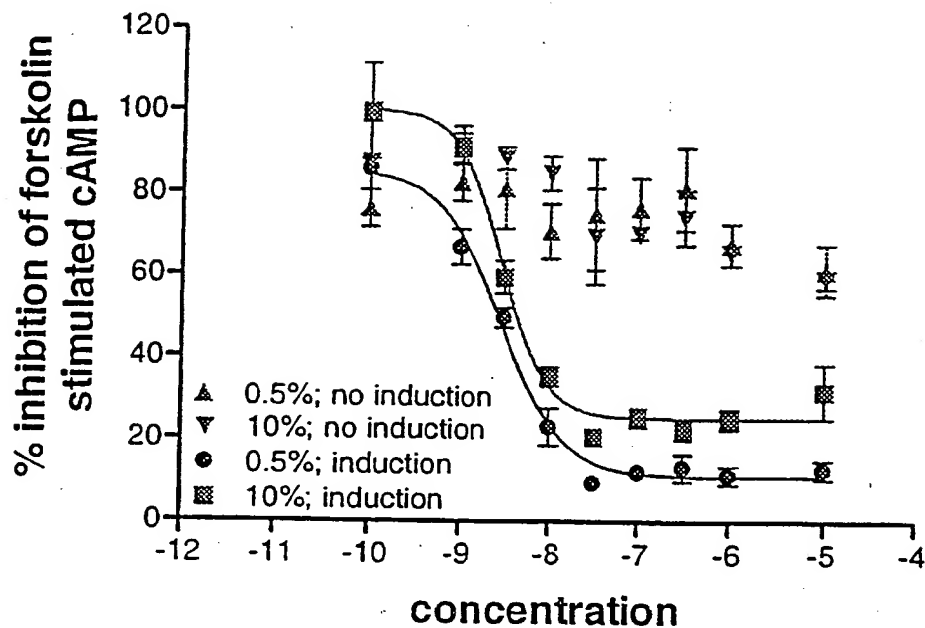


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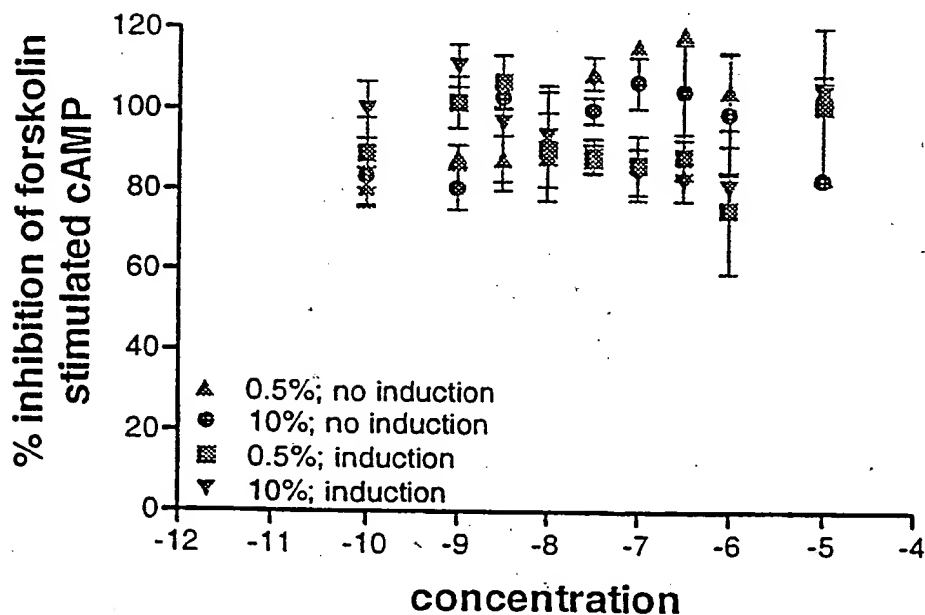


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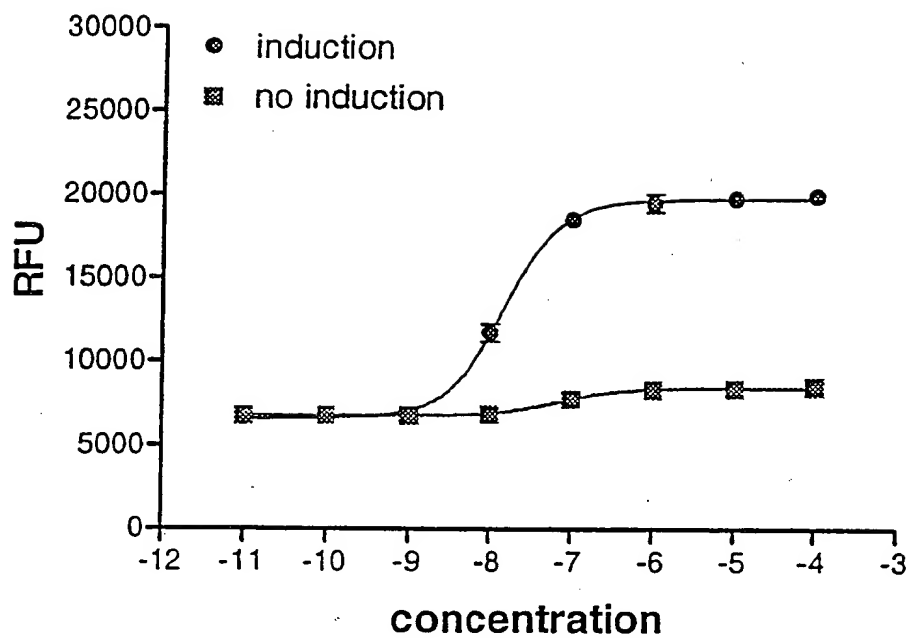
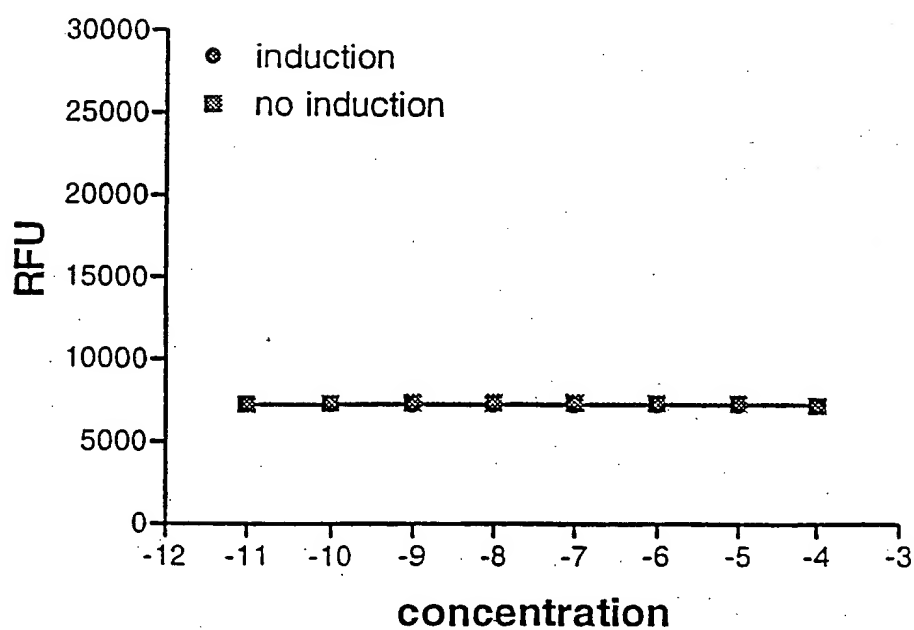


Figure 12b



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994

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&lt;211&gt; 966

&lt;212&gt; DNA

&lt;213&gt; Human

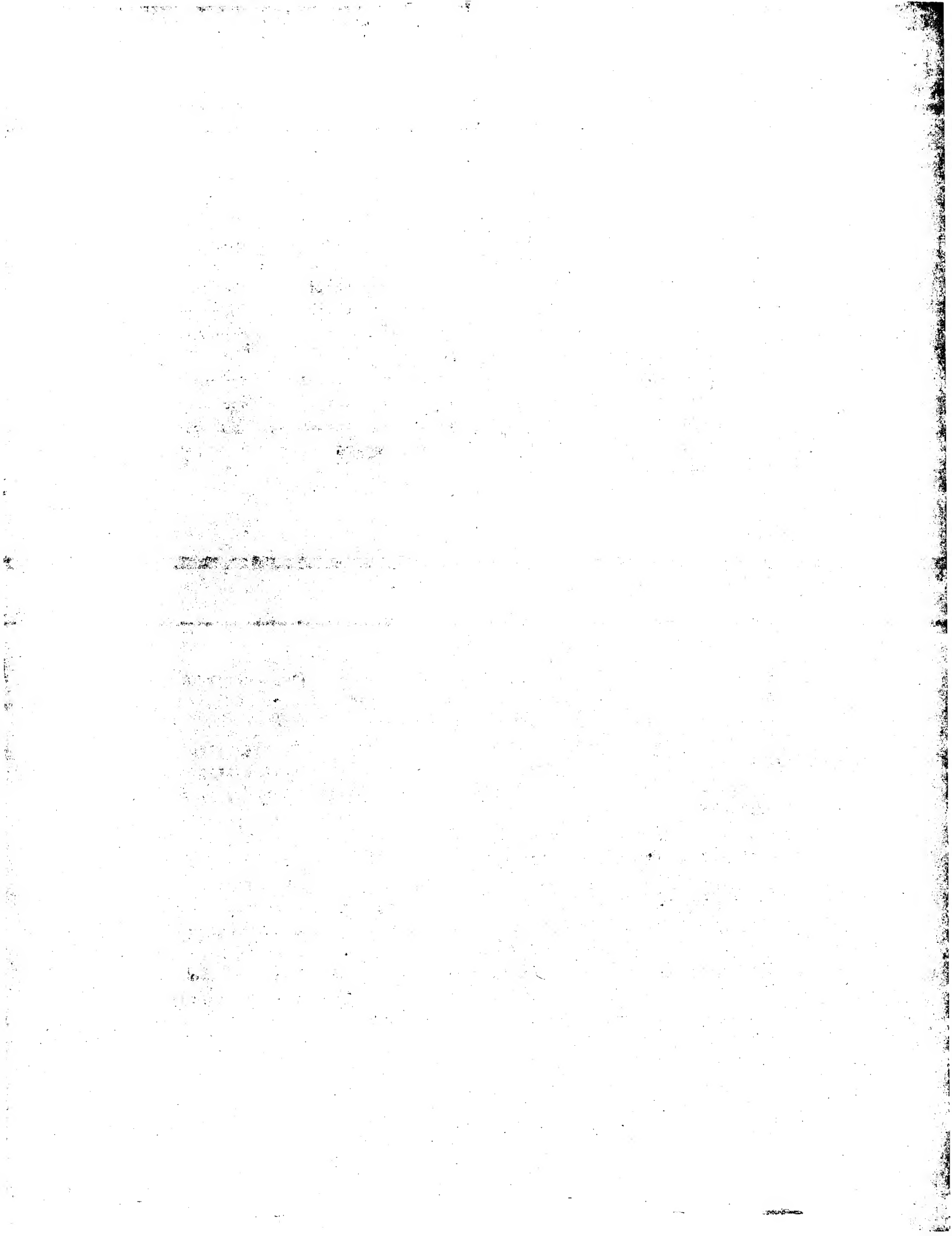
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cagtga

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966



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(54) Title: ADENINE BINDING G PROTEIN COUPLED RECEPTORS

(57) Abstract: The invention provides nucleic acid encoding a G protein coupled receptor (adenine binding GPCR) with specificity for adenine, isolated adenine binding GPCR, assay and treatment methods utilising the same.

## INTERNATIONAL SEARCH REPORT

International Application No

PC/EP 01/03688

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/705 C12N15/12 C12N5/10 A61K48/00 A61K31/711  
 A61K31/52 A61P25/00 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBL, CHEM ABS Data, WPI Data, EP0-Internal, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE AAGENESEQ [Online] ACCESSION NUMBER AAY30162, 26 November 1999 (1999-11-26) AHMAD S ET AL.: "HUMAN DORSAL ROOT RECEPTOR 4 HDNR4" XP002172188 52.761% identity in 326 aa overlap with SEQ ID NO: 2 of the invention; 99.068% identity in 322 aa overlap with SEQ ID NO: 4 of the invention	5-7
X	& WO 99 32519 A (ASTRA PHARMA INC.) 1 July 1999 (1999-07-01) SEQ ID NOS: 9 and 10; SEQ ID NOS: 20 and 21; page 1, line 20 - line 23; claims 21-25,38,40,42 --- -/--	3-16, 21-23



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&amp;" document member of the same patent family

Date of the actual completion of the international search

3 August 2001

Date of mailing of the international search report

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Name and mailing address of the ISA

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Buchet, A

## INTERNATIONAL SEARCH REPORT

International Application No

PC/EP 01/03688

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>DATABASE EMBL [Online]  ACCESSION NUMBER AZ346449,  3 October 2000 (2000-10-03)  DUNN D ET AL.: "MOUSE WHOLE GENOME  SCAFFOLDING WITH PAIRED END READS FROM  10KB PLASMID INSERTS"  XP002172212  81.232% identity in 682 bp with SEQ NO: 1  of the invention; the corresponding  translation product presents 69.469%  identity in 226 aa overlap with SEQ ID NO:  2 of the invention.</p>	3,8-10
X	<p>---  DATABASE EMBL [Online]  ACCESSION NUMBER AC027026,  27 March 2000 (2000-03-27)  BIRREN B. ET AL.: "HOMO SAPIENS CHROMOSOME  11, CLONE RP11-589F4"  XP002172213  67.991% identity in 931 bp overlap with  SEQ ID NO: 1 of the invention; the  corresponding translation product displays  53.11% identity in 337 aa overlap with SEQ  ID NO: 2 of the invention</p>	3,8-10
X	<p>---  DATABASE EMBL [Online]  ACCESSION NUMBER AC023078,  14 February 2000 (2000-02-14)  BIRREN B ET AL.: "HOMO SAPIENS CHROMOSOME  11, CLONE RP11-583F24"  XP002172214  100% identity on the full length with SEQ  NO: 3 (70570-71538), SEQ NO: 5  (27890-28865), SEQ NO: 6 (93615-94580),  SEQ NO: 7 (305-1265), SEQ NO: 8  (123830-124820) and SEQ NO: 9  (47185-48150)</p> <p>-----</p>	1-3,8-10

# INTERNATIONAL SEARCH REPORT

national application No.  
PCT/EP 01/03688

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 11 partially; 17-18 completely  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-10, 12-15, 21-23 completely; 11 partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-10, 12-15, 21-23 completely; 11 partially

concerning mammal G protein coupled receptors (6 from human and 1 from rat) having adenine as a natural ligand: polynucleotides, polypeptides, variants and fragments thereof, recombinant vectors and host cells, methods for identifying modulators and their medical uses.

2. Claims: 19-20

concerning the use of adenine in a medical treatment, possibly associated with purine receptor pathologies.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210.

Continuation of Box I.2

Claims Nos.: 11 partially; 17-18 completely

1) The primer claimed in claim 11 "essentially" consists in 15 to 50 nucleotides of a sequence encoding a polypeptide having at least 50% sequence identity to SEQ ID NO: 2 or 4. This cover a very large number of possible sequences. Among them, a large portion is not searchable: said primers can correspond to the portions diverging from SEQ ID NO: 2 or 4 and therefore have a totally undefined sequence. Only primers corresponding to fragments of SEQ ID NO: 2 or 4 could be searched.

2) Claim 17 refers to a compound merely defined by the method by which it can be identified. In the absence of any characterizing technical feature for unambiguously defining such a product, no meaningful search can be carried out on this aspect of the invention. The same obviously applies to claim 18 which relates to the medical use of such a fully undefined compound.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 01/03688

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9932519	A	01-07-1999	AU 1990499 A	12-07-1999
			BR 9814335 A	10-10-2000
			CN 1284966 T	21-02-2001
			EP 1051434 A1	15-11-2000
			NO 20003221 A	10-08-2000
			PL 341524 A1	23-04-2001
			WO 9932519 A1	01-07-1999
			SK 8752000 A3	18-01-2001
			TR 200001861 T2	21-11-2000
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